The "smoking endotype" of asthma: studies on cigarette smoke-induced, IL-17A dependent neutrophilic inflammation in the bronchial mucosa of asthmatics who smoke

Siew, Leonard Quok Chean

Awarding institution:
King's College London

The "smoking endotype" of asthma: studies on cigarette smoke-induced, IL-17A dependent neutrophilic inflammation in the bronchial mucosa of asthmatics who smoke

Leonard Quok Chean Siew

A thesis submitted to King's College London for the degree of Doctor of Philosophy

2014

MRC & Asthma UK Centre in Allergic Mechanisms of Asthma
Department of Asthma, Allergy and Respiratory Science
King's College London School of Medicine
King's College London

I dedicate this thesis to my mother and father, for without their love, support and sacrifices I would not be here today.

Abstract

Introduction

Many asthmatics smoke, and this has a detrimental effect on the course and control of the disease. The pathophysiological mechanisms through which smoking exerts these effects remain ill defined. The main aims of the studies described in this thesis are to investigate the effects of cigarette smoke on airways structural cells, in particular how it influences the expression of pro-inflammatory and pro-remodelling cytokines, and thereby to understand the role that structural cells may play in the development of Th17/IL-17A mediated neutrophilic inflammation in asthmatics who smoke. A further aim is to show that there is increased vascular remodelling in the airways of asthmatic smokers.

Methods

The neutrophilic and angiogenesis hypotheses were addressed by investigating the effects of cigarette smoke extract (CSE) and IL-17A on human tracheal epithelial cells (HTEpC) and primary bronchial fibroblasts cells in vitro, and also by measuring the expression of these mediators and remodelling changes in bronchial mucosal biopsies from smoking and non-smoking asthmatics in vivo.

Results

There was elevated expression of IL-17A in the bronchial mucosa of asthmatic smokers compared to non-smokers. This was accompanied by elevated expression of IL-6 and IL-8 as well as elevated numbers of neutrophils. In the smoking asthmatics, the expression of IL-17A correlated both with that of IL-8 and with the numbers of neutrophils. Interestingly, the numbers of bronchial mucosal eosinophils also correlated with the expression of IL-8, IL-17A and the numbers of neutrophils. In these mild asthmatic patients there was no difference in the extent of vascular remodelling or numbers of mucosal eosinophils in smokers compared to non-smokers.

Exposure of HTEpC cells to both CSE and IL-17A resulted in increased expression of IL-6 and IL-8 synergistically. The data further suggested that the synergistic

interaction between CSE and IL-17A in HTEpC cells may be mediated by reactive oxygen species (ROS). Co-stimulation of HTEpC cells with CSE, IL-17A and allergens lacking intrinsic protease activity (cat dander and Timothy grass pollen) also increased IL-6 and IL-8 production synergistically. Stimulation of primary human airways fibroblasts with both CSE and IL-17A also resulted in increased expression of IL-6 and VEGF, again with a suggestion of synergy in the effects.

CSE exposure induced HTEpC cells to express VEGF in a concentration-dependent manner. This induction was dependent on MAPK signalling (p38 MAPK, Erk and JNK) and, upstream from this, PI3 kinase-dependent Akt phosphorylation. CSE also induced expression of IL-6, TGF-β1 and VEGF in primary bronchial fibroblasts in a concentration-dependent manner. The induction of IL-6 and VEGF by CSE was found to be dependent on p38 MAPK and ERK signalling, while the induction of TGF-β1 was dependent on ERK and JNK signalling. When primary bronchial fibroblasts were co-stimulated with either Poly I:C or LTA and CSE, there was a synergistic increase in the expression of VEGF.

When HTEpC cells were stimulated with Poly I:C the expression of TSLP was inhibited by CSE, while there was no effect on the expression of IL-6.

Conclusion

Asthmatic smokers have IL-17A mediated neutrophilic inflammation of the airways, which is supported by the effects of CSE interacting with other environmental stimuli (allergens, viruses) on airways epithelial cells and fibroblasts. This evidence supports the categorisation of asthmatic smokers as a specific endotype of asthma.

Statement

All the work presented in the thesis was performed by Leonard Q. C. Siew, with the exception of the immunohistochemical staining which was performed by Miss Celine Wu.

Acknowledgement

Firstly I would like to thank my supervisors Professor Christopher Corrigan for his guidance, support and mentorship during my PhD project, and Professor Tak Lee for having faith in me and giving me the opportunity to read for a PhD.

I would like to give special thanks to Professor James Ritter for his guidance and mentorship while I was writing up my thesis, and to Professor Tim Mant for giving me the opportunity to work at Quintiles Drug Research Unit while reading for my PhD.

Many thanks to Dr Sun Ying and Dr Qiu Meng who gave me tutelage on the various laboratory techniques I had learnt throughout my PhD project. I must also thank Miss Celine Wu for all the immunohistochemical staining she did for me.

I would also like to thank my mother, father and sister for all the love and support they have given me.

I made many great friends during my time at the MRC and Asthma UK Centre in Allergic Mechanisms in Asthma, and I would like to thank everyone for their support in particular Yih-Chih Chan, Yasin Shaifta, Prathap Pillai, Charlotte Farmer, Claire Rooney, Cherylin Reinholtz, Kheem Jones, Rianne Wester, Alick Stephens, Julie Konfortion and Niloufar Safinia.

Lastly but not least I would like to thank the MRC and Asthma UK Centre in Allergic Mechanisms in Asthma, the NIHR Biomedical Research Centre at Guy's and St Thomas' NHS Foundation Trust and King's College London, as well as Quintiles Drug Research Unit for the financial support they have given me.

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Abbreviations

ABPM	Allergic bronchopulmonary mycosis
bFGF	Basic fibroblast growth factor
CD	Cluster of differentiation
CCL	CC chemokine ligands
COPD	Chronic obstructive pulmonary disease
CRP	C-reactive protein
CSF	Colony stimulating factor
CSE	Cigarette smoke extract
CXCL	chemokine (C-X-C motif) ligand
DAB	3,3' -Diaminobenzidine
DAMP	Damage-associated molecular patterns
Dex	Dexamethasone
DMEM	Dulbecco's modified minimum essential medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
dsRNA	Double stranded ribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
eNO	Exhaled nitric oxide
ERK	Extracellular-signal-regulated kinase
EGF	Epidermal growth factor
FEV1	Forced expiratory volume in 1 second
FITC	Fluorescein isothiocyanate
FVC	Forced vital capacity
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GR	Glucocorticoid receptor
GSH	Glutathione
HBSS	Hank's balanced salt solution
HBEC	Human bronchial epithelial cells
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	Horseradish peroxidase
HTEpC	Human tracheal epithelial cells

IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IP	Interferon gamma-induced protein
ISG	Interferon-stimulated genes
JNK	c-Jun N-terminal kinase
LTA	Lipoteichoic acid
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MBP	Major Basic Protein
MCP	Monocyte chemotactic protein
MEK	Mitogen-activated protein kinase kinase
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinases
mRNA	Messenger ribonucleic acid
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide
OVA	Ovalbumin
NaCl	Sodium chloride
NE	Neutrophil elastase
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NHBE	Normal human bronchial epithelial
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PC20	Provocation concentration producing a 20% fall in forced expiratory volume in one second
PDGF	Platelet derived growth factor
PI	Propidium iodide
PI3K	Phosphatidylinositide 3-kinase
Poly I:C	Polyinosinic:polycytidylic acid
RANTES	Regulated on activation, normal T cell expressed and secreted
RORγt	Retinoic acid receptor-related orphan receptor gamma t
ROS	Reactive oxygen species
SAEC	Small airways epithelial cells

SD	Standard deviation
SEM	Standard error mean
SGRQ	St. George's Respiratory Questionnaire
TARC	Thymus and activation regulated chemokine
Th	T helper
TGF	Transforming growth factor
TLR	Toll-like receptors
TNF	Tumor necrosis factor
TSLP	Thymic stromal lymphopoietin
VEGF	Vascular endothelial growth factor
WNT-4	Wingless-type MMTV integration site family

Chapter 1: General Introduction

1 General introduction

Although cigarette smoking is known to predispose to (Vork et al., 2007), and exacerbate asthma (Althuis et al., 1999, James et al., 2005), the practice is still very prevalent, with some estimates suggesting that up to 25% of adolescent asthmatics continue to smoke despite widespread publicity to the contrary (Tyc and Throckmorton-Belzer, 2006, Lenney and Enderby, 2008). Furthermore approximately a quarter of all asthmatics globally are smokers, which coincidentally is also the global prevalence of smoking (To et al., 2012b). For this reason it is particularly important to understand the effects of cigarette smoking on cytokine production and airways remodelling in asthma.

1.1 Asthma

Asthma is characterised by reversible airways obstruction and bronchial hyperresponsiveness. These clinical phenomena are thought to result from inflammatory responses to environmental stimuli acting on the airways. These may be antigen-specific (such as IgE responses to environmental allergens with consequent immediate hypersentivity, and responses to viral infections) or non-specific (responses to stimuli such as environmental tobacco smoke and ozone).

1.1.1 Airways remodelling and angiogenesis in asthma

In asthmatic patients there are characteristic changes in the bronchial mucosa that are collectively termed “remodelling” (increased laying down of interstitial structural proteins such as collagen and fibronectin, angiogenesis, goblet cell hyperplasia, smooth muscle hypertrophy/hyperplasia) (Vignola et al., 1998). These changes have been implicated in contributing to airways hyperresponsiveness and may also contribute to the irreversible airways obstruction that sometimes accompanies asthma and persistent exposure to tobacco smoke (Lambert et al., 1993, Wagers et al., 2004).

1.1.1.1 Reticular basement membrane thickening

Thickening of the reticular layer of the sub-epithelial basement membrane has been well described in the remodelling process of asthmatic airways. There is deposition of collagen types III and V, laminin, fibronectin and tenascin (Roche et al., 1989, Li and Wilson, 1997, Laitinen et al., 1997, Saotome et al., 2003). The thickness of the sub-epithelial basement membrane tenascin layer increases with the degree of eosinophil infiltration and T-cell infiltration of asthmatic airways (Karjalainen et al., 2003). Furthermore, short-term treatment of asthmatics with inhaled corticosteroid, which reduces airways inflammation and improves clinical symptoms, is associated with a reduction in the thickness of this tenascin layer (Laitinen et al., 1997). Thus, lay down of proteins such as tenascin is a valid surrogate of airways remodelling.

1.1.1.2 Airway smooth muscle hypertrophy/hyperplasia

In the airway remodelling process of asthma, there is an increase in airway smooth muscle mass and thickness (Saetta et al., 1991, Carroll et al., 1993). There is evidence that this is due both to hyperplasia (Ebina et al., 1993, Hossain, 1973, Woodruff et al., 2004) and hypertrophy (Benayoun et al., 2003, Ebina et al., 1993) of airway smooth muscle. In addition to their contractile function, airway smooth muscle cells have been shown to secrete cytokines, chemokines, and extracellular matrix components (Howarth et al., 2004).

Previous studies (Benayoun et al., 2003, Pepe et al., 2005) have validated immunohistochemical measurement of surrogates of smooth muscle hyperplasia and hypertrophy that have been found to correlate with disease severity.

1.1.1.3 Angiogenesis

The bronchial mucosa in mild asthmatics has been shown to have increased vascularity (Li and Wilson, 1997). There is an increase in the number of blood vessels and the vascular area, with no significant change in vessel size (Hoshino et al., 2001b, Chetta et al., 2003, Feltis et al., 2006). Long term treatment with high dose inhaled corticosteroids has been shown to reduce both the number of blood vessels and vascular area (Chetta et al., 2003, Hoshino et al., 2001b).

Chetta and colleagues (Chetta et al., 2003) linked these changes with inflammation by showing an association in the asthmatic bronchial mucosa with increased vascularity and the influx of inflammatory cells, particularly eosinophils and mast cells. Furthermore, inhaled corticosteroid therapy produced a dose-dependent reduction of this increased vascularity in concert with the inflammatory cell infiltrate.

The growth factor VEGF (vascular endothelial growth factor) likely plays a key role in this process. Expression of VEGF in the bronchial mucosa and lumen is elevated in asthmatics as compared with controls, and its expression in the mucosa correlates both with both total vessel numbers and vascular area (Feltis et al., 2006, Chetta et al., 2005, Hoshino et al., 2001a). Again, inhaled corticosteroid reduces VEGF expression in concert with vascularity (Chetta et al., 2005).

1.1.2 Airways inflammation in asthma

Airways inflammation in asthma is characterised principally by activation of mucosal CD4+ T cells with a Th2 phenotype and a selective influx of eosinophils (Hamid et al., 1997), but may also involve other inflammatory leukocytes and structural cells such as fibroblasts, epithelial cells and airway smooth muscle cells.

Many cytokines have been implicated in the pathogenesis of asthma, but the products of pro-inflammatory Th2 T cells, including interleukin-4, interleukin-5, interleukin-13 and TNF-α are the most prominent (Chung and Barnes, 1999). In addition to promoting IgE synthesis, these cytokines activate and recruit a number of pro-inflammatory cells hypothesised to be relevant to asthma pathogenesis, and have been shown to induce a variety of remodelling changes within the asthmatic mucosa (Jeffery, 2001). Interferon-γ, a signature product of Th1 T cells, in contrast inhibits Th2 T cell development (Chung and Barnes, 1999), while interleukin-10 and transforming growth factor-β are principal products of T regulatory cells which inhibit both Th1 and Th2 responses (Cools et al., 2007, Fernandez et al., 2008). A reduction in the number of circulating T regulatory cells has been observed in asthmatic patients (Shi et al., 2011, Pietruczuk et al., 2012) and there is increasing evidence that activity of T regulatory cells, both naturally occurring and drug-induced, may be relevant to the prevention or reversal of the asthma inflammatory process (Larche, 2007).

The classical Th2 phenotype and eosinophilic inflammation is seen predominantly in mild asthmatic patients, however in moderate and severe asthmatic patients a neutrophilic inflammation may be seen in the presence or absence of the Th2 phenotype and eosinophilic inflammation. The development of neutrophilic inflammation in moderate/severe asthma is poorly understood and could be the result of either the natural history of the disease process or a sequela of corticosteroid treatment (Wenzel et al., 1997). A possible theory for the development of neutrophilic inflammation in asthma is the involvement of Th17 cells, as the signature cytokines that these cells produce (IL-17A and IL-17F) promote the development of neutrophilia (Laan et al., 1999, Ivanov et al., 2006).

An increase in circulating peripheral blood Th17 cells, with a corresponding increase in peripheral blood IL-17A concentrations, has been found in patients with moderate to severe asthma (Zhao et al., 2011, Shi et al., 2011, Nanzer et al., 2013). An association between asthma control and peripheral blood Th17 cells is also seen in children with asthma, poorer asthma control being associated with higher numbers of Th17 cells (Kerzel et al., 2012). In the bronchial mucosa an increase in IL-17A positive cells has been observed in patients with mild to moderate asthma, but surprisingly not in severe asthma. This increase was not however shown to be associated with airway neutrophilia (Doe et al., 2010). Furthermore IL-23, one of the key cytokines that promotes the differentiation of naïve T cells towards a Th17 phenotype (Chen et al., 2007), has been found to be elevated in the blood of asthmatic children and inversely correlated with pulmonary function (Forced expiratory volume in 1 second) (Ciprandi et al., 2012). Interestingly in asthmatic patients there is an increase in peripheral blood Th17 cells and IL-17A concentrations 24 hours following an allergen challenge (Bajoriuniene et al., 2012).

1.1.3 Interplay of T cells and mediators released by structural cells

T helper cell activation occurs when the T cell receives 2 different signals. The primary activation signal a T helper cell receives from an antigen presenting cell is the presentation of a peptide on the MHC Class II receptor to the T cell receptor (CD3). A co-stimulatory signal via the CD28 receptor is necessary for complete activation of the T cell, and in the absence of this co-stimulatory signal the activated T helper cell

becomes anergic (Damle and Doyle, 1989, Jenkins et al., 1990). The antigen presenting cell also releases a cytokine milieu which directs the differentiation of a naïve T helper cell. The activated naïve T helper cells differentiate to Th1, Th2, iTreg and Th17 cells depending on the cytokine signal (3rd signal) it receives i.e. IL-12, IL-4, TGF-β and TGF-β with IL-6 respectively (Murphy and Stockinger, 2010). Following differentiation it was initially thought that T helper cells are terminally differentiated, however there is now evidence that differentiated T helper cells have a degree of plasticity and with the appropriate cytokine stimuli are able to differentiate from one type of T helper cell to another (Murphy and Stockinger, 2010, Nakayamada et al., 2012). Evidence for in vivo T helper cell plasticity has been demonstrated in the peripheral blood of asthmatic patients (Malmhall et al., 2012).

Increased expression of TSLP has been described in the bronchial epithelial cells of asthmatic patients (Ying et al., 2005). This would indicate that there is a capacity for airway epithelial cells to reinforce the development of Th2 differentiation by the release of thymic stromal lymphopoietin (TSLP) (Soumelis et al., 2002). Furthermore, in response to various inflammatory and environmental stimuli airways epithelial cells (Perng et al., 2006, Murphy et al., 2008), bronchial fibroblast (Wang et al., 2003, Chen et al., 2012) and airways smooth muscle cells (Lee et al., 2006, Michaeloudes et al., 2011) have the capacity to release TGF-β and IL-6. There is also evidence to suggest that both airway epithelial and smooth muscle cells are a source of IL-12 (Hakonarson et al., 1999, Walter et al., 2001). In view of T helper cell plasticity, there is a theoretical possibility that structural cells may be able to influence the differentiation of T helper cells within the vicinity of the inflammatory cytokine milieu they release at the site of inflammation. Not only are structural cells able to release cytokines in response to stimuli, they are also capable of releasing various chemokines, in particular IL-8, CCL11/Eotaxin, CCL17/TARC and RANTES (Teran et al., 1999, Matsukura et al., 2001, Terada et al., 2001, Lordan et al., 2002, Le Bellego et al., 2009). The release of these chemokines results in the influx of myeloid inflammatory cells to the site of inflammation. The interplay between structural cells and the adaptive immune system is poorly understood and structural cells may play a larger role than previously thought in the development of asthmatic inflammation.

Epithelial cells are also a source of a variety of mediators which regulate epithelial growth, angiogenesis and lay down of new proteins within the bronchial mucosa, including vascular endothelial growth factor (VEGF), epithelial growth factor (EGF) and transforming growth factor-β (TGF-β) (Boussat et al., 2000, Chu et al., 2005, Boxall et al., 2006, Thompson et al., 2006), while bronchial fibroblasts maintain and synthesize the extracellular matrix by secreting a variety of structural proteins including collagen types III and V, laminin, fibronectin and tenascin into the basement membrane (Roche et al., 1989, Li and Wilson, 1997, Laitinen et al., 1997, Hoshino et al., 1998a, Saotome et al., 2003, Batra et al., 2003, Degen et al., 2009, Pegorier et al., 2010). They are also a known source of VEGF (Capetandes et al., 2007) the primary growth factor implicated for the increased angiogenesis seen in asthma (Feltis et al., 2006, Chetta et al., 2005, Hoshino et al., 2001a). Finally, these cells are an important source of eicosanoid mediators relevant to asthma, including cysteinyl leukotrienes and prostaglandins (James et al., 2006, Trudeau et al., 2006, Jame et al., 2007, Taira et al., 2007).

1.2 Smoking and asthma

1.2.1 Cigarette smoke and animal "models" of asthma

Animal "models" have produced conflicting data regarding the role of cigarette smoke exposure in asthmatics. On the one hand, Moerloose and colleagues, using a BALB/c mouse model, showed that exposure of ovalbumin sensitised mice to ovalbumin aerosol produced a bronchial luminal eosinophil infiltrate, whereas exposure of the same animals to cigarette smoke independently produced a luminal neutrophil infiltrate (Moerloose et al., 2005). In this particular model, only the two stimuli together produced a significant increase in airways responsiveness to carbachol. They also produced data (Moerloose et al., 2006) suggesting that, in unsensitised mice repeatedly exposed to aerosolised ovalbumin, which does not induce an inflammatory response in unsensitized mice, exposure along with cigarette smoke produced a significantly increased peri-bronchial infiltrate of CD4+ and CD8+ T cells and eosinophils. Cigarette smoke enhances the Th2-driven airway inflammation in the acute phase, as shown above (Moerloose et al., 2006) and by Van Hove and colleagues, and delays but does not prevent the development of inhalation tolerance following repeated exposure to ovalbumin (Van Hove et al., 2008). Nakamura and colleagues (Nakamura et al., 2008) also showed enhancement of the Th2 inflammatory response by cigarette smoke. Using BALB/c mice they showed that cigarette smoke was able to induce the production of the Th2 priming cytokine TSLP. Furthermore they showed that the combined exposure of these mice to both cigarette smoke and ovalbumin enhanced the development of the Th2 inflammatory response that was abrogated by anti-TSLP antibody (Nakamura et al., 2008).

Trimble and colleagues (Trimble et al., 2009) demonstrated that cigarette smoke exposure can act as an adjuvant for ovalbumin sensitization in BALB/c mice, and that the duration of exposure to cigarette smoke did not affect its capacity in this regard. They also showed, however, that prolonged exposure to cigarette smoke following sensitization resulted in an attenuation of the ovalbumin recall response and antigen specific memory (Trimble et al., 2009). These findings were extended by Robays and colleagues (Robays et al., 2009) who showed that in BALB/c mice, the adjuvant effect of cigarette smoke in ovalbumin sensitization is due to an increase in dendritic

cell mediated transport of allergens to mediastinal lymph nodes and a reduction in the numbers of pulmonary plamacytoid dendritic cells (Robays et al., 2009) (which are implicated in the development of inhalation tolerance in BALB/c ovalbumin model of asthma (de Heer et al., 2004)). The ability of cigarette smoke to induce sensitization and enhance the inflammatory response to an allergen has been replicated in a low dose house dust mite allergen BALB/c mouse model of asthma. In this model it was found that co-exposure to cigarette smoke during the initial contact with house dust mite allergen was important in sensitization. Furthermore it also corroborated the findings of Robays and colleagues that cigarette smoke increases dendritic cell mediated transport of allergens to the draining lymph nodes (Lanckacker et al., 2013).

On the other hand, Melgert and colleagues (Melgert et al., 2004), using ovalbumin sensitised C57Bl/6j mice, showed that inhalation challenge of these mice with cigarette smoke along with aerosolised ovalbumin significantly reduced the associated eosinophil infiltrate and increase in bronchial hyperresponsiveness induced by challenge with aerosolised ovalbumin alone. Furthermore Thatcher and colleagues (Thatcher et al., 2008), using ovalbumin sensitised BALB/c mice showed that cigarette smoke was able to suppress the development of a Th2 inflammatory response following challenge with ovalbumin. This suppression was not apparent when they reduced the exposure of the mice to cigarette smoke by 80%. Mishra and colleagues (Mishra et al., 2008) also showed suppression of the Th2 inflammatory response following exposure to a component of cigarette smoke. They pre-treated Brown Norway rats with nicotine prior to ragweed allergen sensitisation and challenge. They showed that nicotine pre-treatment was able to suppress the Th2 inflammatory response to ragweed following inhalation challenge (Mishra et al., 2008). Cigarette smoke, despite attenuating the Th2 inflammatory response in allergen sensitized mice, is associated with an increase in the changes associated with airways remodelling. Botelho and colleagues (Botelho et al., 2011) showed, using house dust mite sensitized BALB/c mice, that cigarette smoke, despite attenuating bronchial eosinophilia, B cell activation and serum IgE concentrations, resulted in an increase in subepithelial collagen deposition. Hizume and colleagues (Hizume et al., 2012) corroborated these findings using ovalbumin sensitized BALB/c mice, and

extended them by showing increased expression of IFN-γ, VEGF and GM-CSF following co-exposure to cigarette smoke and ovalbumin.

These findings are likely protocol dependent and are difficult to extrapolate to humans except as an illustration that smoking and allergen-induced inflammation may interact to influence clinical features of disease.

1.2.2 Effects of smoking on asthma in humans

Exposure to cigarette smoke has been shown to be a key environmental stimulus increasing symptoms (Althuis et al., 1999, Boulet et al., 2006, Eisner and Iribarren, 2007, Chaudhuri et al., 2008, Cerveri et al., 2012, Thomson et al., 2013) and accelerating the decline of lung function in asthmatics (James et al., 2005), with demonstrable reduction in mean airways diameter (Boulet et al., 2006). Cigarette smoke exposure has also been shown to induce corticosteroid insensitivity in asthmatic patients, evident by a reduction in response to treatment with corticosteroids in smoking asthmatics (Chalmers et al., 2002, Chaudhuri et al., 2003). Furthermore smoking cessation has been shown to result in an improvement in asthma symptoms and control (To et al., 2012a). These observations are consistent with the hypothesis that smoking enhances inflammatory and remodelling changes in asthma. This hypothesis is also supported indirectly by studies showing higher total induced sputum inflammatory cells in smoking as compared with non-smoking asthmatics (Chalmers et al., 2001, James et al., 2005, Boulet et al., 2006), with a particular prominence of neutrophils (with a corresponding elevation in interleukin-8 (Chalmers et al., 2001)) which is more characteristic of severe, persistent disease in non-smokers (Kikuchi et al., 2005, Sun et al., 2005).

Cigarette smoking has been shown to be able to reduce exhaled nitric oxide (eNO), a marker of airways inflammation, to an extent comparable with inhaled steroids, in steroid naïve asthmatics (Verleden et al., 1999). Furthermore, in asthmatic smokers, it was found that while eNO was reduced there was an elevation of exhaled hydrogen peroxide (a marker of oxidative stress) (Horvath et al., 2004). The suppression of eNO despite the presence of oxidative stress in the airways could be explained by the finding of increased expression of arginase I and ornithine decarboxylase in the

airways epithelium and smooth muscle bundles in smoking asthmatics, while the expression of inducible nitric oxide synthase (iNOS) was unchanged (both arginase I and iNOS compete for the same substate, L-arginine) (Bruch-Gerharz et al., 2003, Bergeron et al., 2007).

The potential immunomodulatory effects of cigarette smoke in human asthmatics are evident from demonstrations of the reduction of CD83+ mature dentritic cells and CD20+ mature B-lymphocyte numbers (Tsoumakidou et al., 2007), the elevation of interleukin-8 (IL-8) mRNA, interferon-γ (IFN-γ) and neutrophil counts (St-Laurent et al., 2008), and the presence of squamous cell metaplasia and "remodelling" of the airway epithelium (St-Laurent et al., 2008, Broekema et al., 2009) in the bronchial mucosa in asthmatic smokers compared with non-smoking asthmatics. Elevated concentrations of IL-6, IL-7, IL-12 and eotaxin were found in induced sputum of mild asthmatic smokers compared to non-smokers (Krisiukeniene et al., 2009, Spears et al., 2013). Dexamethasone treatment was shown to result in an increase in sputum IL-17 and interferon-α in mild asthmatic smokers but not in asthmatic non-smokers (Spears et al., 2013). Furthermore the tight junctions between bronchial epithelial cells from asthmatic patients are more sensitive to disruption following exposure to cigarette smoke compared to those in healthy controls (Xiao et al., 2011).

Despite these observations there have been few systematic studies comparing the amount and nature of the bronchial mucosal inflammatory infiltrate in asthmatic smokers and non-smokers, and those that do exist suggest that, with the exception of neutrophils, there are few striking differences in cellular infiltration and Th2 cytokine expression (St-Laurent et al., 2008, Broekema et al., 2009), although they did not examine differences in vascular remodelling or the expression of IL-17A, the signature cytokine of Th17 cells (Laan et al., 1999, Ivanov et al., 2006). Furthermore, the physiological and immunological response to inhaled allergen challenge in mild asthmatic smokers has been found to be similar to that of mild asthmatic non-smokers (Meghji et al., 2011). These findings are contrary to the clinical evidence (Althuis et al., 1999, Chaudhuri et al., 2003, James et al., 2005, Boulet et al., 2006, Eisner and Iribarren, 2007, Chaudhuri et al., 2008, Cerveri et al., 2012, Thomson et al., 2013) which suggest that cigarette smoking not only alters the inflammatory profile of

asthma but also affects airway remodelling in asthma. The effects of cigarette smoking on remodelling and inflammation in asthma are thus still unclear.

We suspect that smoking may interact with asthma by:

1. Acting as a non-specific stimulus for bronchial hyperresponsiveness
2. Accelerating decline in lung function (not necessarily by interfering with asthma)
3. Inducing corticosteroid resistance
4. Inducing novel inflammatory changes of its own which may be relevant to asthma symptomatology

The precise mechanism(s) by which smoking interacts with asthma and the weight of these individual potential interactions in asthma control, symptomology and disease progression are yet to be elucidated.

1.3 Endotyping of asthma

The current Th2/eosinophilic model of airways inflammation that we use to understand asthma is a very good model to understand the disease processes on going in patients with mild to moderate asthma. This model however does not fit very well with severe asthma, cough variant asthma and exercise induced asthma. Anderson, having recognised the heterogeneity of asthma, proposed that by classifying asthma into endotypes one would be able better to understand the functional and pathological processes on going in asthma (Anderson, 2008). A consensus on the endotypes used to characterise asthma is yet to be finalised; the currently proposed endotypes of asthma are aspirin sensitive asthma, allergic bronchopulmonary mycosis (ABPM), allergic asthma (adults), asthma-predictive indices positive preschool wheezer, severe late-onset hypereosinophilic, asthma in cross country skiers, early onset allergic, persistent eosinophilia, obese female and neutrophilic (Lotvall et al., 2011, Wenzel, 2012).

Both asthmatic smokers and severe asthmatics have similar clinical characteristics: both have poorly controlled asthma symptoms, frequent exacerbations and accelerated deterioration in lung function, are resistant to corticosteroid treatment and demonstrate a neutrophilic inflammation in the airways (Carmichael et al., 1981, Wenzel et al., 1997, Althuis et al., 1999, Chalmers et al., 2001, Chalmers et al., 2002, Chaudhuri et al., 2003, James et al., 2005, Kikuchi et al., 2005, Sun et al., 2005, Boulet et al., 2006, Hew et al., 2006, Eisner and Iribarren, 2007, Bhavsar et al., 2008, Chaudhuri et al., 2008, BTS and SIGN, 2012, Cerveri et al., 2012, GINA, 2012, Thomson et al., 2013). In view of this asthmatic smokers could potentially be classified under the neutrophilic endotype proposed by Wenzel (Wenzel, 2012), with the caveat that smoking-induced changes may be epiphenomenal.

1.4 Conclusion

Asthma is understood to be associated with airways inflammation and "remodelling". The inflammation is characteristic and associated with elevated expression of Th2-type cytokines and a relatively selective eosinophil influx (Hamid et al., 1997, Chung and Barnes, 1999). Although evidence is largely circumstantial, this inflammation is thought to be at least partly responsible for the cardinal clinical features of asthma (airways obstruction and bronchial hyperresponsiveness) (Lambert et al., 1993, Wagers et al., 2004). Further changes collectively known as "remodelling" (new protein laydown, angiogenesis, mucous hypertrophy, smooth muscle hypertrophy/hyperplasia) are also thought to contribute to the pathophysiology of asthma, including the generation of irreversible airways obstruction, although again the evidence is largely circumstantial (Vignola et al., 1998). Key mediators which are implicated in airways inflammation are the cytokines interleukin-4, interleukin-5, interlukin-13 and TSLP(Chung and Barnes, 1999, Ying et al., 2005); and those implicated in airways "remodelling" are VEGF (Hoshino et al., 2001a), EGF, TGF-β (Boxall et al., 2006) and eicosanoids (Harizi et al., 2008).

Cigarette smoking has well defined clinical affects in asthma, including predisposition to asthma (Vork et al., 2007), exacerbation of symptoms (Althuis et al., 1999, Chaudhuri et al., 2008) and acceleration of decline in lung function (James et al., 2005). This suggests that smoking interacts with asthmatic inflammation and/or remodelling, augmenting it and/or superimposing new changes. It is important to characterise these changes and their mechanisms so that new therapeutic approaches can be delineated. It is unrealistic and demeaning to suggest that this problem will be solved simply by smoking cessation, given the numbers of asthmatics who smoke. Furthermore, such studies will provide further insight into mechanisms of smoking-induced airways changes in non-asthmatics.

The programme of work presented in this thesis represents a concurrent in vitro/in vivo approach to analysis of the effects of cigarette smoke in asthma. Existing studies are few, small and not definitive.

1.5 Hypothesis

1.5.1 Neutrophilia hypothesis

Cigarette smoke stimulates structural cells to produce pro-inflammatory cytokines that support the development and superimposition of Th17/IL-17A mediated neutrophilic inflammation on the allergic Th2/eosinophil mediated inflammation seen in asthma (Figure 1):

- Cigarette smoke extract stimulates structural cells to produce IL-6, Transforming Growth Factor-β1 (TGF-β1), IL-17A and IL-17F.
- Cigarette smoke extract is able to decrease the spontaneous release of TSLP by human tracheal epithelial cells.
- Cigarette smoke extract is able to enhance the pro-inflammatory cytokines produced by IL-17A stimulation of structural cells.
- Reactive oxygen species may be involved in the mechanism underlying the enhancement of pro-inflammatory cytokines produced by IL-17A stimulation of structural cells.
- Aeroallergens are able to modulate the synergistic/additive enhancement seen with co-stimulation with IL-17A and cigarette smoke extract of structural cells.
- Bacterial and viral infections are able to enhance the expression of the pro-inflammatory cytokines induced by cigarette smoke extract on structural cells.
- Cigarette smoke extract is able to attenuate the production of TSLP produced by TLR3 stimulation of structural cells.
- There is increased expression of IL-6 and IL-17A in the bronchial mucosa of asthmatic smokers compared to non-smokers.
- Airways neutrophilia seen in asthmatic smokers is due to Th17/IL-17A mediated inflammation.

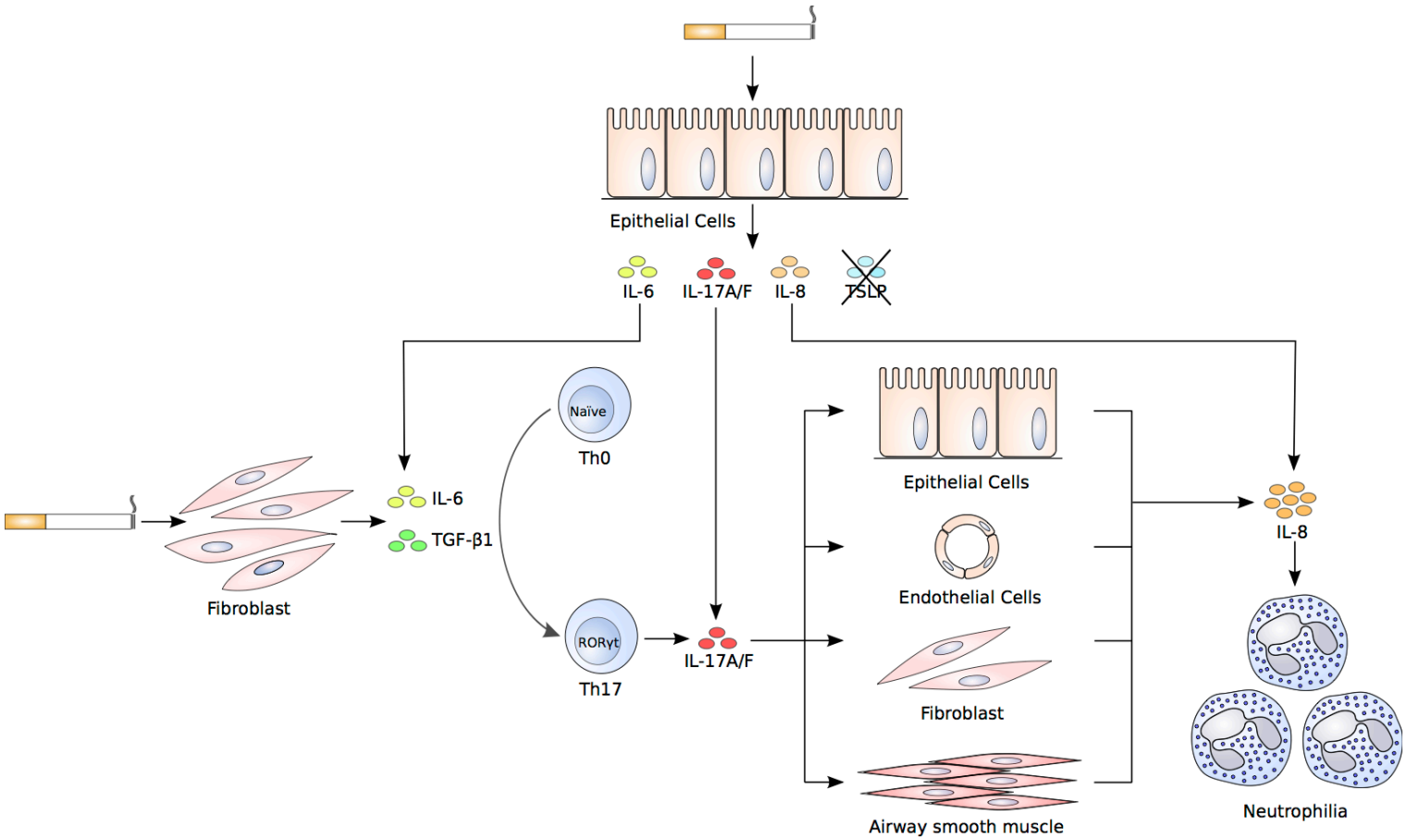

Figure 1: Neutrophilia Hypothesis

Cigarette smoke stimulates structural cells to produce pro-inflammatory cytokines that support the development and superimposition of Th17/IL-17A mediated neutrophilic inflammation on the allergic Th2/eosinophil mediated inflammation seen in asthma.

1.5.2 Angiogenesis hypothesis

Cigarette smoke stimulates airways structural cells to produce pro-remodelling cytokines that support the development of airways remodelling in particular angiogenesis (Figure 2):

- Cigarette smoke extract stimulates structural cells to produce Vascular Endothelial Growth Factor (VEGF), Basic Fibroblast Growth Factor (bFGF), Epithelial Growth Factor (EGF) and Platelet Derived Growth Factor (PDGF)
- Cigarette smoke extract is able to enhance production of the pro-remodelling cytokines produced by IL-17A stimulation of structural cells.
- Bacterial and viral infections are able to enhance the expression of the pro-remodelling cytokines induced by cigarette smoke extract on structural cells.
- There is increased expression of VEGF in the bronchial mucosa of asthmatic smokers compared to non-smokers.
- There is increased angiogenesis in the bronchial mucosa of asthmatic smokers compared to non-smokers.

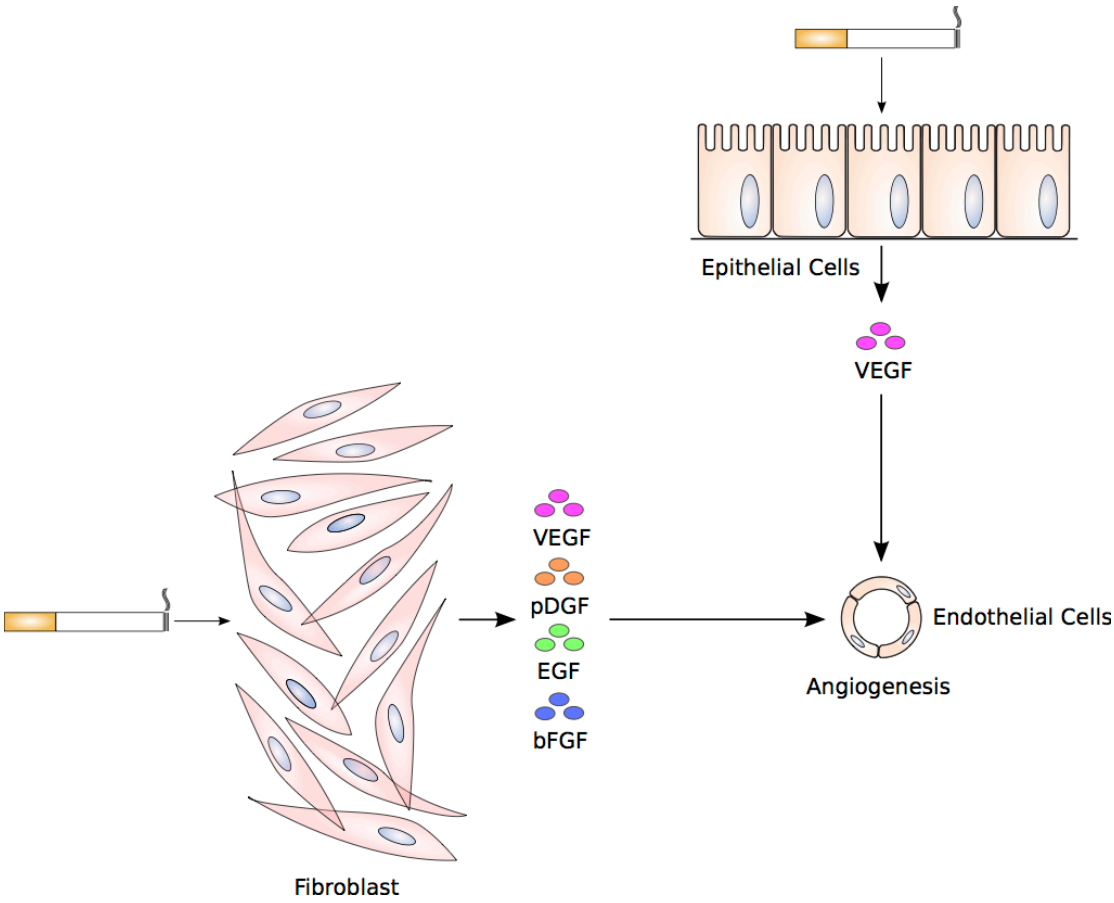

Figure 2: Angiogenesis Hypothesis

Cigarette smoke stimulates structural cells to produce pro-remodelling cytokines that support the development of airways remodelling in particular angiogenesis.

1.6 Aims and objectives

The main aims of this thesis are to investigate the effect of cigarette smoke on airways structural cells, in particular its effects on the expression of pro-inflammatory and pro-remodelling cytokines, and to understand the role that structural cells play in the development of neutrophilic inflammation seen in asthmatic smokers and its influence on the development of Th17/IL-17A mediated neutrophilic inflammation. We also aim to show that there is increased vascular remodelling in asthmatic smokers.

The neutrophilic and angiogenesis hypotheses have been addressed by investigating the effects of cigarette smoke extract, alone and in combination with other environmental stimuli on human tracheal epithelial cells and primary pulmonary fibroblasts cells in vitro, and also by measuring the expression of these mediators and remodelling changes in bronchial mucosal biopsies from smoking and non-smoking asthmatics in vivo.

Chapter 2: Materials and methods

2 Materials and methods

2.1 Cigarette smoke extract (CSE) preparation

Cigarette smoke extract (CSE) was prepared based on a modification of the method of Carp and Janoff (Carp and Janoff, 1978). Two full-strength Marlboro cigarettes (filters removed; Marlboro Red, Class A cigarette, Tar 10 mg, Nicotine 0.8 mg; Phillip Morris USA, Richmond, VA) were combusted and the smoke yielded bubbled through 50 ml of culture medium. A 50 ml syringe (Cat No 300865, BD Plastipak, BD Becton Dickinson UK Ltd, Oxford, UK) was connected to a 3-way stopcock (Cat No 394995, BD Connecta, BD Becton Dickinson UK Ltd, Oxford, UK). One of the other 2 arms of the stopcock was connected to a cigarette via a holder, a 1000 µl unfiltered pipette tip with the tip cut off, and the last arm was connected to a glass Pasteur pipette (Cat D810, John Poulten Ltd, Barking, UK) submerged in a beaker containing culture medium (Figure 3 and Figure 4).

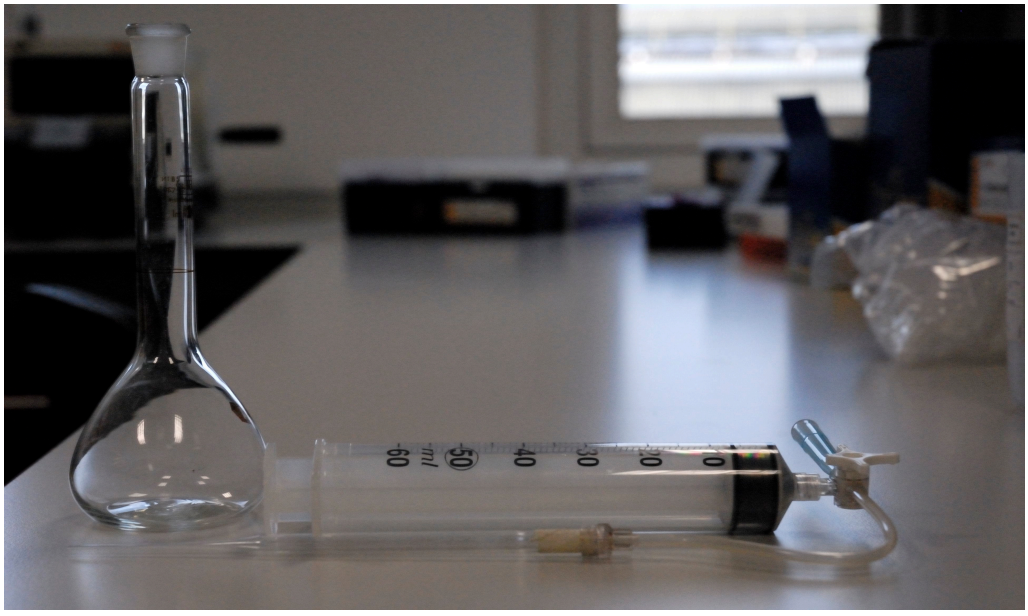

Figure 3. Cigarette Smoke Preparation Device

Figure 4. Cigarette Smoke Extract Preparation Device

In a fume cupboard, each cigarette was lit and cigarette smoke drawn into the 50 ml syringe (to the 60 ml mark) over 10 seconds; following this the cigarette smoke was bubbled into the culture medium. This solution represents '100%' strength. The resulting medium was then sterilized by passing through a 0.20 μm filter (Cat No 16534, Sartorius Stedim UK Ltd, Epsom, UK). The medium was diluted to the required strength and used within 1 hour of preparation (Walters et al., 2005, Oltmanns et al., 2008).

2.2 Fibreoptic bronchoscopy

Fibreoptic bronchoscopy was performed in accordance with the British Thoracic Society Guidelines on diagnostic flexible bronchoscopy (2001, Du Rand et al., 2013). Subjects were sedated with a combination of Midazolam (2-10 mg, non-proprietary, Hameln pharmaceuticals Ltd, Glouchester, UK) and Alfentanyl (200-1000 μg, non-proprietary, Hameln pharmaceuticals Ltd, Glouchester, UK). Instillagel (Lignocaine 2% Gel, CliniMed Ltd, High Wycombe, UK) was used to anaesthetise the nasopharynx while Xylocaine spray (Lignocaine 10%, AstraZeneca UK Ltd, Luton, UK) was used to anaesthetise the oropharynx. The fibreoptic bronchoscope Olympus BF-XT40 (Olympus Keymed, Southend on Sea, UK) was introduced either through the oropharynx or nasopharynx, following which lignocaine 4% (160-240 mg, Guy's and St Thomas' Foundation NHS Trust, London, UK) was used to anaesthetise the vocal cords and laryngopharynx, and lignocaine 2% (80-160 mg, B. Braun Melsungen AG, Melsungen, Germany) was used to anaesthetise the bronchial tree. 10 to 12 endobronchial biopsies were taken using the Olympus FB-35C-1 forceps (Olympus Keymed, Southend on Sea, UK) from the right lower lobe and right middle lobe sub-carinas. Brushings were taken using the Olympus BC-202D-2010 disposable cytology brush (Olympus Keymed, Southend on Sea, UK) from the trachea and the right main bronchus.

2.2.1 Paraformaldehyde fixed endobronchial biopsies

Endobronchial biopsies were fixed in 4% paraformaldehyde (Cat No158127-2.5KG, Sigma-Aldrich, St. Louis, MO) for 2 hours, then transferred to 15% Sucrose (Cat No S9378-1KG, Sigma-Aldrich, St. Louis, MO) in Phosphate Buffered Saline (Cat P4417-100TAB, Sigma-Aldrich, St. Louis, MO) and stored overnight at 4^{o}C. The following day the biopsies were mounted in Optimum Cutting Temperature (O.C.T.) compound (Cat No 361603E, VWR International Ltd, Lutterworth, UK), snap frozen in isopentane (Cat No 103616V, VWR International Ltd, Lutterworth, UK) cooled in liquid nitrogen and stored wrapped in foil in a desiccated atmosphere at –80ºC until used (Ying et al., 2005, Jeffery et al., 2003).

2.2.2 Primary bronchial fibroblast isolation

Primary bronchial fibroblast cells were established by outgrowing from explants according to the method described by Jordana et. al. (Jordana et al., 1988) and Vancheri et. al. (Vancheri et al., 2000). Primary bronchial fibroblasts were grown from normal non-asthmatic bronchial tissue obtained from subjects using endobronchial biopsy. The biopsies were harvested under the approval of London – London Bridge Research Ethics Committee (formally Guy's Research Ethics Committee) (REC reference 08/H0804/87) and Guy's and St Thomas' NHS Foundation Trust Research and Development Department (R&D number RJ1 08/0234). The biopsies were chopped into pieces less than 1 mm^3 and washed once with phosphate-buffered saline (PBS) and twice with Dulbecco's modified minimum essential medium (DMEM) (Cat No 31885-049 Gibco, Life Technologies Ltd, Paisley, UK), containing 10% foetal bovine serum (FBS, Cat No 10082-147, Gibco, Life Technologies Ltd, Paisley, UK), 1% antibiotic-antimycotic (Amphotericin B/streptomycin/penicillin, Cat No 15240-062, Gibco, Life Technologies Ltd, Paisley, UK); 3-5 pieces of washed biopsies were then plated in a 6 well tissue culture plate, with one biopsy placed in each well (Cat No 140675, Nunc, Thermo Fisher Scientific, Rochester, NY). 2 ml of DMEM were added and the tissue was incubated at 37°C with 5% CO2. The medium was changed every 4 days. When a monolayer of fibroblast-like cells covered the bottom of the well, the explant tissue was removed, and the cells were then trypsinized and passaged. Aliquots of cells were frozen and stored in liquid nitrogen.

2.3 Fibroblast Culture

2.3.1 Primary bronchial fibroblasts

Primary bronchial fibroblasts were rescued from liquid nitrogen storage and cultured in a T-75 culture flask (Cat No 156499, Nunc, Thermo Fisher Scientific, Rochester, NY) at 37^{o}C, 5% CO_2 in Dulbecco's modified minimum essential medium (DMEM) (Cat No 31885-049 Gibco, Life Technologies Ltd, Paisley, UK), containing 2 mM L-glutamine, 1% penicillin–streptomycin (Cat No 15140-122, Gibco, Life Technologies Ltd, Paisley, UK), and 10% foetal bovine serum (FBS, Cat No 10082-147, Gibco, Life Technologies Ltd, Paisley, UK).

Primary bronchial fibroblasts were passaged when growth in the T-75 culture flask reached 70-80% confluence. At each passage the cells were split and then cultured in 2 x T-75 culture flasks. As primary bronchial fibroblasts adhere to the base of the flask, following decanting of the culture medium and washing of the cells with Hank's balanced salt solution (HBSS, Cat No 14170-138, Gibco, Life Technologies Ltd, Paisley, UK), 0.05% trypsin with 0.02% EDTA (Cat No 25300-054 Gibco, Life Technologies Ltd, Paisley, UK) was added to the T-75 culture flask to form a cell suspension. When the cells were in suspension, culture medium was then added, in a 1:1 ratio, to stop the action of trypsin. The cell suspension was then centrifuged at 200g, room temperature for 5 minutes, the supernatant decanted, and cells re-suspended in culture medium. This cell suspension was then split evenly and added to 2 x T-75 culture flask containing culture medium and incubated at 37^{o}C in 5% CO_2 incubator.

Cells from passage 4 and 5 were used in the experiments. The cells were passaged from T-75 culture flasks into 12 well tissue culture plates (Cat No 150628, Nunc, Thermo Fisher Scientific, Rochester, NY). Following trypsinization, a trypan blue cell count was performed on the cell suspension. The concentration of the cells in the suspension was then adjusted in order for 100,000 live cells to be plated per well, in a volume of 1 ml for 12 well tissue culture plates. Even distribution of cells to each tissue culture plate well was ensured by gentle agitation of the cell suspension while plating the cells. The cells were then incubated at 37^{o}C in a 5% CO_2 incubator.

When they had just reached 100% confluence, the monolayers of cells in 12 well tissue culture plates were stimulated with various culture conditions. Basal medium used was DMEM (Cat No 31885-049 Gibco, Life Technologies Ltd, Paisley, UK), containing 2 mM L-glutamine, 1% penicillin–streptomycin (Cat No 15140-122, Gibco, Life Technologies Ltd, Paisley, UK), 1% foetal bovine serum (FBS, Cat No 10082-147, Gibco, Life Technologies Ltd, Paisley, UK) and 25 mM HEPES (H3537-100ML, Sigma-Aldrich, St. Louis, MO).

2.3.1.1 CSE concentration response curves

Monolayers of primary bronchial Fibroblasts were exposed to 1 ml per well of the following experimental cell culture medium conditions.

1. CSE 0% in DMEM (Negative control)
2. CSE 0.63% in DMEM
3. CSE 1.25% in DMEM
4. CSE 2.5% in DMEM
5. CSE 5.0% in DMEM
6. CSE 10% in DMEM
7. CSE 20% in DMEM
8. IL-1β 1 ng/ml (Cat No 200-01B PeproTech EC Ltd. London, UK) in DMEM (Positive control)

CSE was diluted in basal medium and reagents added in order to obtain the experimental conditions.

Monolayers of primary bronchial fibroblasts were cultured in experimental conditioned cell culture medium for 24 hrs, 48 hrs and 72 hrs. Following 24 hrs, 48 hrs and 72 hrs of culture the cell culture medium was collected, centrifuged and the cell free supernatants stored at -80 ^{o}C prior to analysis. Cellular viability was assessed visually by trypan blue exclusion.

2.3.1.2 Dexamethasone suppression cultures

Monolayers of primary bronchial fibroblasts were exposed to 1 ml per well of the following experimental cell culture medium conditions.

1. CSE 0% in DMEM
2. CSE 2.5% in DMEM
3. CSE 2.5% and 10^{-6} M Dexamethasone (Cat No D8893, Sigma-Aldrich, St. Louis, MO) in DMEM
4. CSE 2.5% and 10^{-7} M Dexamethasone in DMEM
5. CSE 2.5% and 10^{-8} M Dexamethasone in DMEM
6. 10^{-6} M Dexamethasone in DMEM
7. 10^{-7} M Dexamethasone in DMEM
8. 10^{-8} M Dexamethasone in DMEM
9. IL-1β 1 ng/ml (Cat No 200-01B PeproTech EC Ltd. London, UK) in DMEM (Positive control)
10. Ethanol BP (Pharmacy Department, Guy's and St Thomas' Foundation NHS Trust, London UK) 1/50 dilution in DMEM (Vehicle control for 10^{-6} M Dexamethasone in DMEM)
11. Ethanol BP 1/500 dilution in DMEM (Vehicle control for 10^{-7} M Dexamethasone in DMEM)
12. Ethanol BP 1/5000 dilution in DMEM (Vehicle control for 10^{-8} M Dexamethasone in DMEM)

CSE was diluted in basal medium and reagents added in order to obtain the experimental conditions. Of note dexamethasone was added from stock dissolved in ethanol.

Monolayers of primary bronchial fibroblasts were cultured in experimental conditioned cell culture medium for 24 hrs and 72 hrs. Following 24 hrs and 72 hrs of culture the cell culture medium was collected, centrifuged and the cell free supernatants stored at -80 ^{o}C prior to analysis. Cellular viability was assessed visually by trypan blue exclusion.

2.3.1.3 Lipoteichoic acid (LTA) and polyinosine-polycytidylic acid (Poly I:C) stimulation of fibroblasts

Monolayers of primary bronchial fibroblasts were exposed to 1 ml per well of the following experimental cell culture medium conditions.

1. DMEM
2. 0.1 μg/ml LTA (Cat No tlrl-pslta, InvivoGen, San Diego, CA) in DMEM
3. 0.5 μg/ml LTA in DMEM
4. 1 μg/ml LTA in DMEM
5. 5 μg/ml LTA in DMEM
6. 0.01 μg/ml Poly I:C (Cat No tlrl-pic, InvivoGen, San Diego, CA) in DMEM
7. 0.05 μg/ml Poly I:C in DMEM
8. 0.1 μg/ml Poly I:C in DMEM
9. 0.5 μg/ml Poly I:C in DMEM

CSE was diluted in basal medium and reagents added in order to obtain the experimental conditions. Purified lipoteichoic acid from Staphylococcus aureus (LTA) (Cat No tlrl-pslta, InvivoGen, San Diego, CA) and purified polyinosinic:polycytidylic acid (Poly I:C) (Cat No tlrl-pic, InvivoGen, San Diego, CA) was used in these experiments.

Monolayers of primary bronchial fibroblasts were cultured in experimental conditioned cell culture medium for 24 hrs and 72 hrs. Following 24 hrs and 72 hrs of culture the cell culture medium was collected, centrifuged and the cell free supernatants stored at -80 ^{o}C prior to analysis. Cellular viability was assessed visually by trypan blue exclusion.

2.3.1.4 CSE and lipoteichoic acid (LTA) co-stimulation of fibroblasts

Monolayers of primary bronchial fibroblasts were exposed to 1 ml per well of the following experimental cell culture medium conditions.

1. CSE 0% in DMEM

2. CSE 2.5% in DMEM
3. CSE 2.5% and 0.1 μg/ml LTA (Cat No tlrl-pslta, InvivoGen, San Diego, CA) in DMEM
4. CSE 2.5% and 0.5 μg/ml LTA in DMEM
5. CSE 2.5% and 1 μg/ml LTA in DMEM
6. 0.1 μg/ml LTA in DMEM
7. 0.5 μg/ml LTA in DMEM
8. 1 μg/ml LTA in DMEM

CSE was diluted in basal medium and reagents added in order to obtain the experimental conditions. Purified lipoteichoic acid from Staphylococcus aureus (Cat No tlrl-pslta, InvivoGen, San Diego, CA) was used in these experiments.

Monolayers of primary bronchial fibroblasts were cultured in experimental conditioned cell culture medium for 24 hrs and 72 hrs. Following 24 hrs and 72 hrs of culture the cell culture medium was collected, centrifuged and the cell free supernatants stored at -80 ^{o}C prior to analysis. Cellular viability was assessed visually by trypan blue exclusion.

2.3.1.5 CSE and polyinosine-polycytidylic (Poly I:C) acid co-stimulation of fibroblasts

Monolayers of primary bronchial fibroblasts were exposed to 1 ml per well of the following experimental cell culture medium conditions.

1. CSE 0% in DMEM
2. CSE 2.5% in DMEM
3. CSE 2.5% and 0.01 μg/ml Poly I:C (Cat No tlrl-pic, InvivoGen, San Diego, CA) in DMEM
4. CSE 2.5% and 0.05 μg/ml Poly I:C in DMEM
5. CSE 2.5% and 0.1 μg/ml Poly I:C in DMEM
6. 0.01 μg/ml Poly I:C in DMEM
7. 0.05 μg/ml Poly I:C in DMEM
8. 0.1 μg/ml Poly I:C in DMEM

CSE was diluted in basal medium and reagents added in order to obtain the experimental conditions. Purified polyinosinic:polycytidylic acid (Poly I:C) (Cat No tlrl-pic, InvivoGen, San Diego, CA) was used in these experiments.

Monolayers of primary bronchial fibroblasts were cultured in experimental conditioned cell culture medium for 24 hrs and 72 hrs. Following 24 hrs and 72 hrs of culture the cell culture medium was collected, centrifuged and the cell free supernatants stored at -80 ^{o}C prior to analysis. Cellular viability was assessed visually by trypan blue exclusion.

2.3.1.6 CSE and interleukin-17A co-stimulation of fibroblasts

Monolayers of primary bronchial fibroblasts were exposed to 1 ml per well of the following experimental cell culture medium conditions.

1. CSE 0% in DMEM
2. CSE 2.5% in DMEM
3. CSE 2.5% and 0.1 ng/ml recombinant human IL-17A (Cat No 200-17, PeproTech EC Ltd. London, UK) in DMEM
4. CSE 2.5% and 1 ng/ml recombinant human IL-17A in DMEM
5. CSE 2.5% and 10 ng/ml recombinant human IL-17A in DMEM
6. 0.1 ng/ml recombinant human IL-17A in DMEM
7. 1 ng/ml recombinant human IL-17A in DMEM
8. 10 ng/ml recombinant human IL-17A in DMEM

CSE was diluted in basal medium and reagents added in order to obtain the experimental conditions.

Monolayers of primary bronchial fibroblasts were cultured in experimental conditioned cell culture medium for 24 hrs and 72 hrs. Following 24 hrs and 72 hrs of culture the cell culture medium was collected, centrifuged and the cell free supernatants stored at -80 ^{o}C prior to analysis. Cellular viability was assessed visually by trypan blue exclusion.

2.3.1.7 CSE signal transduction experiments

Monolayers of primary bronchial fibroblasts were exposed to 1 ml per well of the following experimental cell culture medium conditions.

1. CSE 0% in DMEM
2. CSE 2.5% in DMEM
3. CSE 2.5% and 2.5 μM BAY11-7082 (Cat no 196870, Calbiochem, EMD Chemicals, Inc., Gibbstown, NJ) in DMEM
4. CSE 2.5% and 5 μM SB203580 (Cat no 559398, Calbiochem, EMD Chemicals, Inc., Gibbstown, NJ) in DMEM
5. CSE 2.5% and 5 μM PD98059 (Cat no 10006726, Cayman Chemical Company, Ann Arbor, MI) in DMEM
6. CSE 2.5% and 2.5 μM SP600125 (Cat no S5567, Sigma-Aldrich, St. Louis, MO) in DMEM
7. CSE 2.5% and 10 μM U0126 (Cat no 662005, Calbiochem, EMD Chemicals, Inc., Gibbstown, NJ) in DMEM
8. CSE 2.5% and 10 μM LY294002 (Cat no 440204, Calbiochem, EMD Chemicals, Inc., Gibbstown, NJ) in DMEM
9. Dimethyl Sulfoxide (DMSO, Cat no D5879, Sigma-Aldrich, St. Louis, MO) 1/500 dilution (Vehicle control) in DMEM
10. DMSO 1/1000 dilution (Vehicle control) in DMEM
11. DMSO 1/2000 dilution (Vehicle control) in DMEM
12. DMSO 1/40000 dilution (Vehicle control) in DMEM

CSE was diluted in basal medium and reagents added in order to obtain the experimental conditions.

Monolayers of primary bronchial fibroblasts were cultured in experimental conditioned cell culture medium for 24 hrs and 72 hrs. Following 24 hrs and 72 hrs of culture the cell culture medium was collected, centrifuged and the cell free supernatant stored at -80 ^{o}C prior to analysis. Cellular viability was assessed visually by trypan blue exclusion.

2.4 Epithelial cell culture

2.4.1 Human tracheal epithelial cell culture

Human tracheal epithelial cells (HTEpC) were purchased from PromoCell (Cat No C-12644, PromoCell GmbH, Heidelberg, Germany). HTEpC cells were rescued from liquid nitrogen storage and cultured in a bovine type I collagen (PureCol, Cat No 5005, Advanced BioMatrix, Inc., San Diego, CA) coated T-75 culture flask (Cat No 156499, Nunc, Thermo Fisher Scientific, Rochester, NY) at 37^{o}C, 5% CO_2 in airway epithelial cell growth medium containing bovine pituitary extract 0.004 ml/ml, epidermal growth factor 10 ng/ml, insulin 5 μg/ml, hydrocortisone 0.5 μg/ml, epinephrine 0.5 μg/ml, triiodo-L-thyronine 6.7 ng/ml, holo-transferrin 10 μg/ml, retinoic acid 0.1 ng/ml (Cat No C-21160, PromoCell GmbH, Heidelberg, Germany) with 1% antibiotic-antimycotic (Amphotericin B/streptomycin/penicillin, Cat No 15240-062, Gibco, Life Technologies Ltd, Paisley, UK). HTEpC cells were passaged when growth in the T-75 culture flask reached 70-80% confluence. At each passage the cells were split and then cultured in 2 to 3 bovine type I collagen coated T-75 culture flasks.

HTEpC cells adhere to the base of the flask. To passage, following decanting of the culture medium and washing of the cells with Hank's balanced salt solution (HBSS, Cat No 14170-138, Gibco, Life Technologies Ltd, Paisley, UK), 0.05% trypsin with 0.02% EDTA (Cat No 25300-054 Gibco, Life Technologies Ltd, Paisley, UK) was added to the T-75 culture flask to form a cell suspension. When the cells were in suspension, trypsin neutralisation solution (Cat no 17075-029, Life Technologies Ltd, Paisley, UK) was then added, in a 1:1 ratio, to stop the action of trypsin. The cell suspension was then centrifuged at 200g, room temperature for 5 minutes, the supernatant decanted, and cells re-suspended in culture medium. This cell suspension was then split evenly and added to 2 to 3 T-75 culture flasks containing culture medium and incubated at 37^{o}C in 5% CO_2 incubator.

Cells from passage 4 and 5 were used in the experiments. The cells were passaged from T-75 culture flasks into 6 well and 12 well bovine type I collagen (PureCol, Cat No 5005, Advanced BioMatrix, Inc., San Diego, CA) coated tissue culture plates (Cat

No 140675; Cat No 150628, Nunc, Thermo Fisher Scientific, Rochester, NY). Following trypsinization, a trypan blue cell count was performed on the cell suspension. The concentrations of the cells in the suspensions were then adjusted in order for 40,000 to 50,000 live cells and 80,000 to 100,000 live cells to be plated per well, in a volume of 1 ml and 2 mls, for 12 well and 6 well bovine type I collagen coated tissue culture plates respectively. Even distribution of cells to each tissue culture plate well was ensured by gentle agitation of the cell suspension while plating the cells. The cells were then incubated at 37^{o}C in 5% CO_2 incubator.

When the cells had just reached 100% confluence in the 6 well and 12 well tissue culture plates, the airway epithelial cell growth culture medium was changed to airway epithelial cell starvation medium containing bovine pituitary extract 0.004 ml/ml, epidermal growth factor 10 ng/ml, insulin 5 µg/ml, triiodo-L-thyronine 6.7 ng/ml, holo-transferrin 10 µg/ml, (Cat No C-21160, PromoCell GmbH, Heidelberg, Germany) with 1% antibiotic-antimycotic (Amphotericin B/streptomycin/penicillin, Cat No 15240-062, Gibco, Life Technologies Ltd, Paisley, UK) and 25 mM HEPES (H3537-100ML, Sigma-Aldrich, St. Louis, MO). The monolayers were starved for a period of 24 hours following which they were stimulated with various conditions.

2.4.1.1 CSE concentration response curves

Monolayers of HTEpC were exposed to 1 ml per well (12 well tissue culture plates) of the following experimental cell culture medium conditions.

1. CSE 0% in airway epithelial cell starvation medium
2. CSE 0.04% in airway epithelial cell starvation medium
3. CSE 0.08% in airway epithelial cell starvation medium
4. CSE 0.16% in airway epithelial cell starvation medium
5. CSE 0.31% in airway epithelial cell starvation medium
6. CSE 0.63% in airway epithelial cell starvation medium
7. CSE 1.25% in airway epithelial cell starvation medium
8. CSE 2.5% in airway epithelial cell starvation medium
9. IL-1β 1 ng/ml (Cat No 200-01B PeproTech EC Ltd. London, UK) in airway epithelial cell starvation medium

CSE was diluted in airway epithelial cell starvation medium and reagents added in order to obtain the experimental conditions.

Monolayers of HTEpC were cultured in experimental conditioned cell culture medium for 24 hrs, 48 hrs and 72 hrs. Following 24 hrs, 48 hrs and 72 hrs of culture the cell culture medium was collected, centrifuged and the cell free supernatant stored at -80 ^{o}C prior to analysis. Cellular viability was assessed visually by trypan blue exclusion.

2.4.1.2 CSE and interleukin-17A co-stimulation

Monolayers of HTEpC were exposed to 1 ml per well (12 well tissue culture plates) of the following experimental cell culture medium conditions.

1. CSE 0% in airway epithelial cell starvation medium
2. CSE 0.16% in airway epithelial cell starvation medium
3. CSE 0.16% and 0.1 ng/ml recombinant human IL-17A (Cat No 200-17, PeproTech EC Ltd. London, UK) in airway epithelial cell starvation medium
4. CSE 0.16% and 1 ng/ml recombinant human IL-17A in airway epithelial cell starvation medium
5. CSE 0.16% and 10 ng/ml recombinant human IL-17A in airway epithelial cell starvation medium
6. 0.1 ng/ml recombinant human IL-17A in airway epithelial cell starvation medium
7. 1 ng/ml recombinant human IL-17A in airway epithelial cell starvation medium
8. 10 ng/ml recombinant human IL-17A in airway epithelial cell starvation medium

CSE was diluted in airway epithelial cell starvation medium and reagents added in order to obtain the experimental conditions.

Monolayers of HTEpC were cultured in experimental conditioned cell culture medium for 24 hrs. Following 24 hrs of culture the cell culture medium was collected, centrifuged and the cell free supernatant stored at -80 ^{o}C prior to analysis. Cellular viability was assessed visually by trypan blue exclusion.

2.4.1.3 Inhibition of CSE and interleukin-17A co-stimulation with glutathione

Monolayers of HTEpC were exposed to 1 ml per well (12 well tissue culture plates) of the following experimental cell culture medium conditions.

1. CSE 0% in airway epithelial cell starvation medium
2. CSE 0.16% in airway epithelial cell starvation medium
3. CSE 0.16% and glutathione 1 mM (Cat No G6013-5G, Sigma-Aldrich, St. Louis, MO) in airway epithelial cell starvation medium
4. 10 ng/ml recombinant human IL-17A (Cat No 200-17, PeproTech EC Ltd. London, UK) in airway epithelial cell starvation medium
5. 10 ng/ml recombinant human IL-17A and glutathione 1 mM (in airway epithelial cell starvation medium
6. CSE 0.16% and 10 ng/ml recombinant human IL-17A in airway epithelial cell starvation medium
7. CSE 0.16%, 10 ng/ml recombinant human IL-17A and glutathione 1 mM in airway epithelial cell starvation medium

CSE was diluted in airway epithelial cell starvation medium and reagents added in order to obtain the experimental conditions. IL-17A and glutathione were also diluted in airway epithelial cell starvation medium and reagents added in order to obtain the experimental conditions.

Monolayers of HTEpC were cultured in experimental conditioned cell culture medium for 24 hrs. Following 24 hrs of culture the cell culture medium was collected, centrifuged and the cell free supernatant stored at -80 ^{o}C prior to analysis. Cellular viability was assessed visually by trypan blue exclusion.

2.4.1.4 CSE, interleukin-17A and allergen extract co-stimulation

Monolayers of HTEpC were exposed to 1 ml per well (12 well tissue culture plates) of the following experimental cell culture medium conditions.

1. CSE 0% in airway epithelial cell starvation medium
2. CSE 0.16% in airway epithelial cell starvation medium
3. 10 ng/ml recombinant human IL-17A (Cat No 200-17, PeproTech EC Ltd. London, UK) in airway epithelial cell starvation medium
4. 5000 SQ-U/ml Felis domesticus Aquagen SQ (Cat No ALK(555), ALK-Abello A/S, Horsholm, Denmark)
5. 5000 SQ-U/ml Phleum Pratense Aquagen SQ (Cat No ALK(225), ALK-Abello A/S, Horsholm, Denmark)
6. CSE 0.16% and 10 ng/ml recombinant human IL-17A in airway epithelial cell starvation medium
7. CSE 0.16% and 5000 SQ-U/ml Felis domesticus Aquagen SQ in airway epithelial cell starvation medium
8. CSE 0.16% and 5000 SQ-U/ml Phleum Pratense Aquagen SQ in airway epithelial cell starvation medium
9. 10 ng/ml recombinant human IL-17A and 5000 SQ-U/ml Felis domesticus Aquagen SQ in airway epithelial cell starvation medium
10. 10 ng/ml recombinant human IL-17A and 5000 SQ-U/ml Phleum Pratense Aquagen SQ in airway epithelial cell starvation medium
11. CSE 0.16%, 10 ng/ml recombinant human IL-17A and 5000 SQ-U/ml Felis domesticus Aquagen SQ in airway epithelial cell starvation medium
12. CSE 0.16%, 10 ng/ml recombinant human IL-17A and 5000 SQ-U/ml Phleum Pratense Aquagen SQ in airway epithelial cell starvation medium

CSE was diluted in airway epithelial cell starvation medium and reagents added in order to obtain the experimental conditions. IL-17A and allergen extracts were also diluted in airway epithelial cell starvation medium and reagents added in order to obtain the experimental conditions.

Monolayers of HTEpC were cultured in experimental conditioned cell culture medium for 24 hrs. Following 24 hrs of culture the cell culture medium was collected, centrifuged and the cell free supernatant stored at -80 ^{o}C prior to analysis. Cellular viability was assessed visually by trypan blue exclusion.

2.4.1.5 CSE and polyinosine-polycytidylic co-stimulation

Monolayers of HTEpC were exposed to 1 ml per well (12 well tissue culture plates) of the following experimental cell culture medium conditions.

1. CSE 0% in airway epithelial cell starvation medium
2. CSE 0.16% in airway epithelial cell starvation medium
3. CSE 0.16% and 0.1 μg/ml Poly I:C (Cat No tlrl-pic, InvivoGen, San Diego, CA) in airway epithelial cell starvation medium
4. CSE 0.16% and 1 μg/ml Poly I:C in airway epithelial cell starvation medium
5. CSE 0.16% and 10 μg/ml Poly I:C in airway epithelial cell starvation medium
6. 0.1 μg/ml Poly I:C in airway epithelial cell starvation medium
7. 1 μg/ml Poly I:C in airway epithelial cell starvation medium
8. 10 μg/ml Poly I:C in airway epithelial cell starvation medium

CSE was diluted in airway epithelial cell starvation medium and reagents added in order to obtain the experimental conditions.

Monolayers of HTEpC were cultured in experimental conditioned cell culture medium for 24 hrs. Following 24 hrs of culture the cell culture medium was collected, centrifuged and the cell free supernatant stored at -80 ^{o}C prior to analysis. Cellular viability was assessed visually by trypan blue exclusion.

2.4.1.6 CSE signal transduction experiments

Monolayers of HTEpC were exposed to 1 ml per well (12 well tissue culture plates) of the following experimental cell culture medium conditions.

1. CSE 0% in airway epithelial cell starvation medium

2. CSE 0.16% in airway epithelial cell starvation medium
3. CSE 0.16% and 2.5 μM BAY11-7082 (Cat no 196870, Calbiochem, EMD Chemicals, Inc., Gibbstown, NJ) in airway epithelial cell starvation medium
4. CSE 0.16% and 5 μM SB203580 (Cat no 559398, Calbiochem, EMD Chemicals, Inc., Gibbstown, NJ) in airway epithelial cell starvation medium
5. CSE 0.16% and 5 μM PD98059 (Cat no 10006726, Cayman Chemical Company, Ann Arbor, MI) in airway epithelial cell starvation medium
6. CSE 0.16% and 2.5 μM SP600125 (Cat no S5567, Sigma-Aldrich, St. Louis, MO) in airway epithelial cell starvation medium
7. CSE 0.16% and 10 μM U0126 (Cat no 662005, Calbiochem, EMD Chemicals, Inc., Gibbstown, NJ) in airway epithelial cell starvation medium
8. CSE 0.16% and 10 μM LY294002 (Cat no 440204, Calbiochem, EMD Chemicals, Inc., Gibbstown, NJ) in airway epithelial cell starvation medium
9. Dimethyl Sulfoxide (DMSO, Cat no D5879, Sigma-Aldrich, St. Louis, MO) 1/500 dilution (Vehicle control) in airway epithelial cell starvation medium
10. DMSO 1/1000 dilution (Vehicle control) in airway epithelial cell starvation medium
11. DMSO 1/2000 dilution (Vehicle control) in airway epithelial cell starvation medium
12. DMSO 1/40000 dilution (Vehicle control) in airway epithelial cell starvation medium

CSE was diluted in airway epithelial cell starvation medium and reagents added in order to obtain the experimental conditions.

Monolayers of HTEpC were cultured in experimental conditioned cell culture medium for 24 hrs. Following 24 hrs of culture the cell culture medium was collected, centrifuged and the cell free supernatant stored at -80 ^{o}C prior to analysis. Cellular viability was assessed visually by trypan blue exclusion.

2.5 Enzyme-linked immunosorbent assay (ELISA)

2.5.1 VEGF, IL-6, IL-8, bFGF, IL-17A, IL-17F, TSLP, PDGF-BB

Capture antibody was diluted in PBS and plated on to a BD Falcon 96-well ELISA Microplate (Cat No 353279, BD Biosciences, San Diego, CA), 100 µl/well, then the plate sealed and incubated overnight at room temperature. The following capture antibodies were plated for the respective ELISA measurements:

1. VEGF – 0.5 µg/ml antigen-affinity purified rabbit anti-hVEGF antibody (Cat No 900-K10, PeproTech EC Ltd. London, UK)
2. IL-6 – 1 µg/ml antigen-affinity purified goat anti-hIL-6 antibody (Cat No 900-K16, PeproTech EC Ltd. London, UK)
3. IL-8 – 0.5 µg/ml antigen-affinity purified rabbit anti-hIL-8 antibody (Cat No 900-K18, PeproTech EC Ltd. London, UK)
4. IL-17A – 0.5 µg/ml antigen-affinity purified goat anti-hIL-17A antibody (Cat No 900-K84, PeproTech EC Ltd. London, UK)
5. IL-17F – 1 µg/ml antigen-affinity purified rabbit anti-hIL-17F antibody (Cat No 900-K277, PeproTech EC Ltd. London, UK)
6. TSLP – 1 µg/ml antigen-affinity purified rabbit anti-hTSLP antibody (Cat No 900-K334, PeproTech EC Ltd. London, UK)
7. bFGF – 1 µg/ml antigen-affinity purified rabbit anti-human bFGF antibody (Cat No 900-K08, PeproTech EC Ltd. London, UK)
8. PDGF-BB – 1 µg/ml antigen-affinity purified rabbit anti-hPDGF-BB antibody (Cat No 900-K04, PeproTech EC Ltd. London, UK)

The wells were then aspirated and washed with 300 µl/well of wash buffer, 0.05% Tween-20 in PBS (Cat No: P3563-10PAK, Sigma-Aldrich, St. Louis, MO) 4 times. The wells were then blocked with 200 µl/well of 1% bovine serum albumin (BSA) in PBS (1:10 dilution of 10% BSA solution, Cat No DY995, R&D Systems, Inc. Minneapolis, MN), and the plates sealed and incubated at room temperature for 1 hour.

The wells were then aspirated and washed with 300 µl/well of wash buffer 4 times. 100 µl/well of standard and 100 µl/well of sample were then added and the plates sealed and incubated at room temperature for 2 hours, except for IL-8 ELISA where 50 µl/well of sample were plated.

The following recombinant proteins were used as standards for the respective ELISA measurements:

1. VEGF –recombinant human VEGF (Cat No 900-K10, PeproTech EC Ltd. London, UK)
2. IL-6 – recombinant human IL-6 (Cat No 900-K16, PeproTech EC Ltd. London, UK)
3. IL-8 – recombinant human IL-8 (Cat No 900-K18, PeproTech EC Ltd. London, UK)
4. IL-17A – recombinant human IL-17A (Cat No 900-K84, PeproTech EC Ltd. London, UK)
5. IL-17F – recombinant human IL-17F (Cat No 900-K277, PeproTech EC Ltd. London, UK)
6. TSLP – recombinant human TSLP (Cat No 900-K334, PeproTech EC Ltd. London, UK)
7. bFGF – recombinant human bFGF (Cat No 900-K08, PeproTech EC Ltd. London, UK)
8. PDGF-BB – recombinant human PDGF-BB (Cat No 900-K04, PeproTech EC Ltd. London, UK)

Standard curves were generated using the following concentrations of recombinant proteins: 2000 pg/ml, 1000 pg/ml, 500 pg/ml, 250 pg/ml, 125 pg/ml, 62.5 pg/ml, 31.3 pg/ml, 15.6 pg/ml, 7.8 pg/ml, 3.9 pg/ml and blank, diluted in 0.05% Tween-20, 0.1% BSA in PBS. In the case of bFGF the following concentrations were used: 4000 pg/ml, 2000 pg/ml, 1000 pg/ml, 500 pg/ml, 250 pg/ml, 125 pg/ml, 62.5 pg/ml, 31.3 pg/ml and blank, diluted in 0.05% Tween-20, 0.1% BSA in PBS.

The wells were then aspirated and washed with 300 µl/well of wash buffer 4 times. Detection antibody was diluted in 0.05% Tween-20, 0.1% BSA in PBS, 100 µl/well were added and the plates sealed and incubated for 2 hours at room temperature.

The following detection antibodies were plated for the respective ELISA measurements:

1. VEGF – 0.25 µg/ml biotinylated antigen-affinity purified rabbit anti-hVEGF antibody (Cat No 900-K10, PeproTech EC Ltd. London, UK)
2. IL-6 – 0.25 µg/ml biotinylated antigen-affinity purified goat anti-hIL-6 antibody (Cat No 900-K16, PeproTech EC Ltd. London, UK)
3. IL-8 – 0.25 µg/ml biotinylated antigen-affinity purified rabbit anti-hIL-8 antibody (Cat No 900-K18, PeproTech EC Ltd. London, UK)
4. IL-17A – 0.25 µg/ml biotinylated antigen-affinity purified goat anti-hIL-17A antibody (Cat No 900-K84, PeproTech EC Ltd. London, UK)
5. IL-17F – 1 µg/ml biotinylated antigen-affinity purified rabbit anti-hIL-17F antibody (Cat No 900-K277, PeproTech EC Ltd. London, UK)
6. TSLP – 0.25 µg/ml biotinylated antigen-affinity purified rabbit anti-hTSLP antibody (Cat No 900-K334, PeproTech EC Ltd. London, UK)
7. bFGF – 1 µg/ml biotinylated antigen-affinity purified rabbit anti-human bFGF antibody (Cat No 900-K08, PeproTech EC Ltd. London, UK)
8. PDGF-BB – 0.25 µg/ml biotinylated antigen-affinity purified rabbit anti-hPDGF-BB antibody (Cat No 900-K04, PeproTech EC Ltd. London, UK)

The wells were then aspirated and washed with 300 µl/well of wash buffer 4 times. 100 µl/well streptavidin-horseradish peroxidase (Cat No DY998, R&D Systems, Inc. Minneapolis, MN) at 1:200 dilution in 1% BSA in PBS, were then added and the plates sealed and incubated for 20 minutes at room temperature.

The wells were then aspirated and washed with 300 µl/well of wash buffer 4 times. Substrate solution, consisting of stabilised hydrogen peroxide (Colour Reagent A, Cat No DY999, R&D Systems, Inc. Minneapolis, MN) and 3, 3', 5, 5' tetramethylbenzidine (Colour Reagent B, Cat No DY999, R&D Systems, Inc.

Minneapolis, MN) in a 1:1 ratio was prepared within 15 minutes prior to use. 100 µl/well substrate solution were then added and the plates incubated for a maximum of 30 minutes in the dark at room temperature.

50 µl/well of stop solution, 2N sulphuric acid (Cat No DY994, R&D Systems, Inc. Minneapolis, MN), were then added. The plates were read using an ELISA reader at 450 nm within 30 minutes. Wavelength correction was set at 578 nm.

2.5.2 TGF-β1

Capture antibody was diluted in PBS and plated on to a BD Falcon 96-well ELISA Microplate (Cat No 353279, BD Biosciences, San Diego, CA), 100 µl/well, then the plate sealed and incubated overnight at room temperature. The following capture antibody was plated for TGF-β1 ELISA measurements:

1. TGF-β1 – 2.0 µg/ml mouse anti-hTGF-β1 antibody (Cat No DY240, R&D Systems, Inc. Minneapolis, MN)

The wells were then aspirated and washed with 300 µl/well of wash buffer, 0.05% Tween-20 in PBS (Cat No: P3563-10PAK, Sigma-Aldrich, St. Louis, MO) 3 times. The wells were then blocked with 300 µl/well of 5% Tween 20 (Cat No 663684B, VWR International Ltd, Lutterworth, UK) in PBS (Cat No P4417-100TAB, Sigma-Aldrich, St. Louis, MO) with 0.05% NaN_3 (Cat No S2002-500G, Sigma-Aldrich, St. Louis, MO), and the plates sealed and incubated at room temperature for 1 hour.

Samples for TGF-β1 ELISA measurements were activated as follows:

1. 40 µl of 1 N HCl (Cat No 320331-2.5L, Sigma-Aldrich, St. Louis, MO) were added to 200 µl of sample and incubated for 10 minutes at room temperature.
2. 40 µl of 1.2 N NaOH (Cat No 102524X, VWR International Ltd, Lutterworth, UK)/0.5 M HEPES (Cat No H7637-100G, Sigma-Aldrich, St. Louis, MO) were added to neutralise the acidified sample.

Samples were assayed immediately following activation.

The wells were then aspirated and washed with 300 µl/well of wash buffer 3 times. 100 µl/well of standard and 100 µl/well of activated sample were then added and the plates sealed and incubated at room temperature for 2 hours.

The following recombinant protein was used as standard for TGF-β1 ELISA measurements:

1. TGF-β1 –recombinant human TGF-β1 (Cat No DY240, R&D Systems, Inc. Minneapolis, MN)

Standard curves were generated using the following concentrations of recombinant TGF-β1: 2000 pg/ml, 1000 pg/ml, 500 pg/ml, 250 pg/ml, 125 pg/ml, 62.5 pg/ml, 31.3 pg/ml, 15.6 pg/ml, 7.8 pg/ml, 3.9 pg/ml and blank, diluted in 1.4% delipidized bovine serum (Reagent Diluent Concentrate 1, Cat No DY997 R&D Systems, Inc. Minneapolis, MN), 0.05% Tween 20 in PBS.

The wells were then aspirated and washed with 300 µl/well of wash buffer 3 times. Detection antibody was diluted in 1.4% delipidized bovine serum, 0.05% Tween 20 in PBS, 100 µl/well were added and the plates sealed and incubated for 2 hours at room temperature

The following detection antibody was plated for TGF-β1 ELISA measurements:

1. TGF-β1 – 0.3 µg/ml biotinylated chicken anti-hTGF-β1 antibody (Cat No DY240, R&D Systems, Inc. Minneapolis, MN)

The wells were then aspirated and washed with 300 µl/well of wash buffer 3 times. 100 µl/well Streptavidin-horseradish peroxidase (Cat No DY998, R&D Systems, Inc. Minneapolis, MN) at 1:200 dilution in 1.4% delipidized bovine serum, 0.05% Tween

20 in PBS, were then added and the plates sealed and incubated for 20 minutes at room temperature.

The wells were then aspirated and washed with 300 µl/well of wash buffer 3 times. Substrate solution, consisting of stabilised hydrogen peroxide (Colour Reagent A, Cat No DY999, R&D Systems, Inc. Minneapolis, MN) and 3, 3', 5, 5' tetramethylbenzidine (Colour Reagent B, Cat No DY999, R&D Systems, Inc. Minneapolis, MN) in a 1:1 ratio was prepared within 15 minutes prior to use. 100 µl/well substrate solution were then added and the plates incubated for a maximum of 30 minutes in the dark at room temperature.

50 µl/well of stop solution, 2 N sulphuric acid (Cat No DY994, R&D Systems, Inc. Minneapolis, MN), were then added. The plates were read using an ELISA reader at 450 nm within 30 minutes. Wavelength correction was set at 578 nm.

2.6 *In vivo* study design

The study was conducted in accordance with Good Clinical Practice, World Medical Association Declaration of Helsinki and departmental standard operating procedures. The study protocol was reviewed and approval received from London – London Bridge Research Ethics Committee (formally Guy's Research Ethics Committee) (REC reference 06/Q0704/175) and Guy's and St Thomas' NHS Foundation Trust Research and Development Department (R&D number RJ1 07/0069).

The overall study design was a cross sectional case control study of asthmatic smokers, with asthmatic non-smokers and healthy non-smokers as controls.

The study was powered as a pilot study, since when we commenced the study there were no suitable publications available which would provide us with data with which to power it. However a power calculation was performed using published means and standard deviations of the airways remodelling study end-points in healthy subjects and asthmatic subjects as follows (data are quoted as the mean/standard deviation):

1. Basement membrane thickness: 9.4 μm ± 1.2 asthma, 8.0 μm ± 0.2 controls (Chetta et al., 2005).
2. Smooth muscle area (per total biopsy): 1.2 mm^2 ± 0.1 asthma, 0.9 mm^2 ± 0.4 controls (James et al., 2002).
3. Number of mucosal blood vessels: 209 vessels/mm^2 ± 54 asthma, 153 vessels/mm^2 ± 23 controls (Chetta et al., 2005).
4. Mean vascular area (% total biopsy area): 5.2% ± 1.6 asthma, 3.0% ± 0.7 controls (Chetta et al., 2005).
5. Submucosal mast cells 65 cells/mm^2 ± 27 asthma, 35 cells/mm^2 ± 9 controls (Chetta et al., 2005).
6. Submucosal eosinophils 101 cells/mm^2 ± 80 asthma, 31 cells/mm^2 ± 22 controls (Chetta et al., 2005).

The size of all the effects shown above is above 1. A sample size of 16 subjects in each of the three subgroups guarantees 80% power ($\alpha = 0.5$) to detect, as statistically significant, standardised differences of size 1 and above in all the outcomes considered.

Subjects were stratified into 3 groups that were defined as follows: steroid naïve mild asthmatic smoker, steroid naïve mild asthmatic non-smoker and non-asthmatic non-smoker control (i.e healthy non-smokers). Taking the above powering exercise into consideration it was decided that we would recruit 20 subjects for each group.

The following definitions were used to qualify asthma and smoking status:

- **Asthma:** Documented history for ≥ 6 months prior to screening, histamine $PC_{20} \leq 8$ mg/ml.
- **Non-Asthma:** Life long absence of relevant symptoms, FEV1 ≥ 80% predicted, histamine $PC_{20} \geq 16$mg/ml.

- **Non-smoker**: No smoking within 12 months of screening, < 0.5 pack year total
- **Smoker:** Currently smoking ≥ 5 cigarettes per week

All subjects recruited into the study had to satisfy the inclusion and exclusion criteria for the study as follows:

Inclusion criteria

- Males and females aged 18 to 55 years inclusive.

Exclusion criteria

- Past or present disease, which as judged by the investigator, might affect the outcome of this study. These diseases include, but are not limited to,

cardiovascular disease, malignancy, hepatic disease, renal disease, haematological disease, neurological disease, endocrine disease or pulmonary disease (including but not confined to chronic bronchitis, emphysema, bronchiectasis or pulmonary fibrosis).

- Subjects must not have any clinically significant deviation from normal in either the general physical examination or laboratory parameters as evaluated by the investigator at the screening visit.
- Pregnant or lactating females.
- History of a respiratory tract infection and/or exacerbation of asthma within 4 weeks of the screening visit.
- History of life-threatening asthma, defined as an asthma episode that required intubation and/or was associated with hypercapnoea, respiratory arrest and/or hypoxic seizures.
- Participation in a study involving research bronchoscopy in the previous 3 months.
- Subject is undergoing allergen desensitisation therapy.
- Any regular medication that may affect the outcome of the trial, other than intermittent, inhaled short-acting beta-agonist.
- Inability to understand or comply with the research protocol.

Subjects were initially recruited from the participant database of the Department of Asthma, Allergy and Respiratory Science, King's College London. We had also employed advertising and circular emails (approved by the research ethics committee) to facilitate recruitment of subjects to the study.

At the screening visit we obtained informed consent before any screening activities were conducted. Subjects who had given their written informed consent had undergone a full assessment of their medical history (including a smoking history), physical examination, vital signs (blood pressure, respirations, oxygen saturation and heart rate), skin prick testing (which may be historical for up to 12 months) and laboratory evaluation for routine haematology and a blood clotting profile. Spirometry was performed and FEV_1, FVC, FEV_1/FVC ratio measured. Bronchial hyper-responsiveness was measured using histamine bronchial challenge and the

provocation concentration of histamine causing a 20% fall in baseline FEV_1 (histamine PC_{20}) calculated.

Within one month of the screening visit, subjects returned for their study visit. They were assessed for adverse events, their medical history reviewed and consent reaffirmed. Vital signs were re-checked and spirometry with reversibility (using 2.5 mg of salbutamol via nebuliser) performed. Subjects were also asked to complete a validated respiratory quality of life questionnaire, the St. George's Respiratory Questionnaire (SGRQ) (Jones et al., 1991).

Fibreoptic bronchoscopy was performed in accordance to the British Thoracic Society guidelines (2001, Du Rand et al., 2013). Endobronchial biopsies were obtained from the subcarina of the 3rd to 7th generation bronchi, with the aim of obtaining 6 biopsies that were suitable for immunohistochemistry assessment (which may entail taking up to 12 biopsies in total).

2.7 Immunohistochemical staining

We had initially used the DAB staining method to stain the endobronchial biopsy sections and this was subsequently changed to the Fast Red staining method as it was easier to identify individual cells which were stained with Fast Red compared to that stained with DAB in particular when staining for soluble cytokines which could produce a diffuse pattern of staining. As CD31 staining localised very well to the endothelium and it was very clear which cells had stained positive, we did not re-stain the endobronchial biopsies for CD31. The numbers of sections stained for CD31 differ from those used to detect other markers because we obtained satisfactory data with DAB and did not re-stain with Fast Red.

2.7.1 DAB immunohistochemical staining

Sections of bronchial biopsies of nominal 6 μm thickness were stained as follows. Slides were thawed following storage in -80^{o}C. The thawed slides were placed in a slide holder and box and washed with PBS (Cat No P4417-100TAB, Sigma-Aldrich, St. Louis, MO). The box containing the slides was filled with enough PBS to cover the slides. This was placed onto a rocker for 10 mins, following which the PBS was discarded. The slides were washed with PBS a further 2 more times for a total of 3 washes.

Excess PBS was removed from around the tissue and the slides were then laid out in a humidified chamber. 60 μl of pre-block solution were then added directly on top of the tissue, which were then incubated at room temperature in the dark for 10 minutes.

The pre-block solution used to block endogenous peroxide activity was 0.3% Hydrogen Peroxide (Cat No H1009-500ML, Sigma-Aldrich, St. Louis, MO) in 0.1% Sodium Azide (Cat No S2002-500G, Sigma-Aldrich, St. Louis, MO).

The slides were then placed in a slide holder and box and washed with PBS. The box containing the slides was filled with enough PBS to cover the slides. This was placed onto a rocker for 5 mins, following which the PBS was discarded. The slides were washed with PBS a further 2 more times for a total of 3 washes.

Excess PBS was removed from around the tissue and the slides were then laid out in a humidified chamber. 10% FBS (Cat No 10082-147, Gibco, Life Technologies Ltd, Paisley, UK) in PBS blocking buffer was then added directly on top of the tissue, ensuring that the tissue was completely covered by solution, and the tissue then incubated at room temperature in the dark for 1 hour.

Excess blocking buffer was then removed from around the tissue and the slides laid out in a humidified chamber. 60 µl of diluted primary antibody were then added directly on top of the tissue, and the tissue then incubated at room temperature in the dark overnight.

The following primary antibody was used:

1. CD31 primary antibody (Monoclonal mouse anti-human, Clone JC70A, Cat No M0823, Dako, Ely, UK) (1 in 100 dilution)

The slides were then placed in a slide holder and box and washed with PBS. The box containing the slides was filled with enough PBS to cover the slides. This was placed onto a rocker for 5 mins, following which the PBS was discarded. The slides were washed with PBS a further 2 more times for a total of 3 washes.

Excess PBS was removed from around the tissue and the slides were then laid out in a humidified chamber. 60 µl of HRP-labelled secondary antibody (Goat anti mouse polyclonal Antibody, Cat No A0168-1ML, Sigma-Aldrich, St. Louis, MO) diluted in PBS (1:100 dilution) were then added directly on top of the tissue, which was then incubated at room temperature in the dark for 30 minutes.

The slides were then placed in a slide holder and box and washed with PBS. The box containing the slides was filled with enough PBS to cover the slides. This was placed onto a rocker for 5 mins, following which the PBS was discarded.

Excess PBS was removed from around the tissue and the slides were then laid out in a dry chamber. 60 µl of freshly made DAB substrate solution (SIGMAFAST™ 3,3′-Diaminobenzidine tablets, Cat No D4293-50SET, Sigma-Aldrich, St. Louis, MO) were then added directly on top of the tissue, which was then incubated at room temperature in the dark. The reaction was then stopped by placing the slides in a slide holder and box and washed with PBS. The box containing the slides was filled with enough PBS to cover the slides. This was placed onto a rocker for 5 mins, following which the PBS was discarded. The slides were washed with PBS a further 2 more times for a total of 3 washes.

Excess PBS was removed from around the tissue and the slides were then laid out in a dry chamber. 60µl of filtered Mayer's Hematoxylin solution (Cat No MHS32-1L, Sigma-Aldrich, St. Louis, MO) was then added directly on top of the tissue, which was then incubated at room temperature for 1 minute. The slides were then placed in a slide holder and box and washed with water. The box containing the slides was filled with water, and running water was passed along the slide holder for at least 15 minutes.

Excess water was removed from around the tissue and the slides were then laid out in a dry chamber and allowed to dry for 1 hour. The slides were then mounted with DPX mounting medium for microscopy (Cat No 360294H, VWR International Ltd, Lutterworth, UK) and covered using a coverslip and left overnight in a dry dark chamber to dry.

The slides were then stored in a slide box and analysed using a Zeiss Vision KS300 system (Carl Zeiss, Gottingen, Germany). This system offers objective, unbiased digital image analysis for the required measurements using a powerful macro language.

Images (x400 magnification) were digitised with a three-chip colour camera, connected to a computer and frame grabber software with appropriate corrections for non-uniform illumination (shading correction) and settings of the black and white camera signal (white balance).

All image analysis and measurements were totally objective but were nevertheless performed by an operator ignorant of the origin of the sections.

Imaging data were computed as follows:

- Discrete inflammatory cells stained with phenotypic markers were recorded as the total cells per unit area of the sections.

2.7.2 Fast red immunohistochemical staining

Sections of bronchial biopsies of nominal 6 μm thickness were stained as follows. Slides were thawed following storage in -80^{o}C. The thawed slides were placed in a slide holder and box and washed with PBS (Cat No P4417-100TAB, Sigma-Aldrich, St. Louis, MO). The box containing the slides was filled with enough PBS to cover the slides. This was placed onto a rocker for 10 mins, following which the PBS was discarded. The slides were washed with PBS a further 2 more times for a total of 3 washes.

Excess PBS was removed from around the tissue and the slides were then laid out in a humidified chamber. 60 μl of diluted primary antibody were then added directly on top of the tissue, which was incubated at room temperature in the dark overnight.

The primary antibody was diluted in 5% human serum (Normal Human Serum, Cat No 31876, Thermo Fisher Scientific, Rochester, NY) in PBS. The following primary antibodies were used:

1. Neutrophil Elastase primary antibody (Monoclonal mouse anti-human, Clone NP57, Cat No M0752, Dako, Ely, UK) (1 in 100 dilution)
2. Major Basic Protein primary antibody (Monoclonal mouse anti-human, Clone BMK13, Cat No ab77842, Abcam, Cambridge, UK) (1 in 30 dilution)
3. Vascular Endothelial Growth Factor primary antibody (Polyclonal rabbit anti-human, Cat No ab46154, Abcam, Cambridge, UK) (1 in 500 dilution)
4. IL-6 primary antibody (Polyclonal rabbit anti-human, Cat No ab6672, Abcam, Cambridge, UK) (1 in 500 dilution)

5. IL-8 primary antibody (Polyclonal goat anti-human, Cat No AF-208-NA, R&D Systems, Inc. Minneapolis, MN) (1 in 500 dilution)
6. IL-17A primary antibody (Polyclonal mouse anti-human, Cat No 14-7179-82, eBioscience, Hatfield, UK) (1 in 200 dilution)

The slides were then placed in a slide holder and box and washed with PBS. The box containing the slides was filled with enough PBS to cover the slides. This was placed onto a rocker for 5 mins, following which the PBS was discarded. The slides were washed with PBS a further 2 more times for a total of 3 washes. Excess PBS was removed from around the tissue and the slides were then laid out in a humidified chamber.

When the species of primary antibody used was either rabbit or goat, a secondary antibody was employed. 60 µl of Alkaline Phosphatase-labelled secondary antibody (Goat anti-Rabbit, Cat No A3687-1ML, Sigma-Aldrich, St. Louis, MO and Rabbit anti-goat, Cat No 4062-1ML, Sigma-Aldrich, St. Louis, MO Alkaline Phosphatase-labelled polyclonal Antibody respectively) diluted in 5% human serum in PBS (1:50 dilution) were added directly on top of the tissue which was then incubated at room temperature in the dark for up to 1 hour.

When the species of primary antibody used was mouse, both secondary and tertiary amplification were employed. 60 µl of secondary antibody (Rabbit anti-mouse IgG antibody, Cat no SAB3701021-2MG, Sigma-Aldrich, St. Louis, MO) diluted in 5% human serum in PBS (1:50 dilution) were then added directly on top of the tissue, which was then incubated at room temperature in the dark for 30 minutes. The slides were then placed in a slide holder and box and washed with PBS. The box containing the slides was filled with enough PBS to cover the slides. This was placed onto a rocker for 5 mins, following which the PBS was discarded. The slides were washed with PBS a further 2 more times for a total of 3 washes. 60 µl of tertiary antibody (Mouse-Alkaline Phosphatase-Anti-Alkaline Phosphatase antibody, Cat No A7827-1ML, Sigma-Aldrich, St. Louis, MO) diluted in 5% human serum in PBS (1:30 dilution) were then added directly on top of the tissue, which was incubated at room temperature in the dark for 30 minutes.

Following the respective amplification steps above, the slides were then placed in a slide holder and box and washed with PBS. The box containing the slides was filled with enough PBS to cover the slides. This was placed onto a rocker for 5 mins, following which the PBS was discarded. The slides were washed with PBS a further 2 more times for a total of 3 washes.

Excess PBS was removed from around the tissue and the slides were then laid out in a dry chamber. 60 µl of freshly made Fast red substrate solution (SIGMAFAST™ Fast Red TR/Naphthol AS-MX tablets, Cat No F4648-50SET Sigma-Aldrich, St. Louis, MO) were then added directly on top of the tissue, which was then incubated at room temperature in the dark. The reaction was then stopped by placing the slides in a slide holder and box and washed with deionised water. The box containing the slides was filled with enough deionised water to cover the slides. This was placed onto a rocker for 5 mins, following which the deionised water was discarded.

Excess deionised water was removed from around the tissue and the slides were then laid out in a dry chamber. 60 µl of filtered Mayer's Hematoxylin solution (Cat No MHS32-1L, Sigma-Aldrich, St. Louis, MO) was then added directly on top of the tissue, and incubated at room temperature for 1 minute. The slides were then placed in a slide holder and box and washed with water. The box containing the slides was filled with water, and running water was passed along the slide holder for at least 15 minutes.

Excess water was removed from around the tissue and the slides were then laid out in a dry chamber and allowed to dry for 1 hour. The slides were then mounted with glycerol gelatin mounting medium for microscopy (GG1-15ML Sigma-Aldrich, St. Louis, MO) and covered using a coverslip and left overnight in a dry dark chamber to dry.

The slides were then stored in a slide box and analysed using a Zeiss Vision KS300 system as described.

Imaging data were computed as follows:

- Discrete inflammatory cells stained with phenotypic markers were recorded as the total cells per unit area of the sections.

2.8 Cell counts and viability assessments

2.8.1 Trypan blue exclusion

Cell counts were performed on cell suspensions using trypan blue 0.4% solution (Cat No: T8154-20ML, Sigma-Aldrich, St. Louis, MO). 10 µl of cell suspension were diluted in trypan blue 0.4% solution in a 1:9 ratio. 10 µl of the trypan blue cell suspension were then placed into each chamber of a Bright-Line Haemocytometer (Cat No: Z359629-1EA, Sigma-Aldrich, St. Louis, MO). Cells in the outer 4 quadrants (4x4 Quadrants) were counted. Within each small square, cells touching the left or bottom lines/sides were counted and those touching the top and right lines/sides were excluded. Viable cells were defined as those excluding trypan blue. The mean numbers of live cells in each outer quadrant, (n) was determined. This indicates the cellular concentration as n x 10^4 cells/ml, from which the total cell count was determined (total cell count = n x 10^4 x dilution factor x volume of cell suspension) and the concentration of the cell suspension adjusted.

Cellular viability was assessed at 24 hrs, 48 hrs and 72 hrs of culture with experimental conditioned cell culture medium. Cellular viability was assessed by trypan blue exclusion and we also monitored cellular confluence as a surrogate measurement of viability.

2.8.2 Annexin V and propidium iodide staining

Apoptosis and cell death were assessed using the FITC Annexin V/Dead Cell Apoptosis Kit (Cat No V13242, Invitrogen, Life Technologies Ltd, Paisley, UK). Primary bronchial fibroblasts were exposed to a concentration series of CSE, as described in section 2.3.1.1. Following 24 hours of culture the cell culture medium was collected and centrifuged. The cell pellet of suspension cells was resuspended in cold HBSS. Adherent cells were detached using 0.05% trypsin with 0.02% EDTA, and resultant cell suspension pooled with the corresponding resuspended cell pellet. The pooled cell suspension was then centrifuged and the supernatant discarded. Cold HBSS was then added to "wash" the cells, by centrifuging and discarding the supernatant. The pooled cells were washed twice with HBSS. Following the final wash the pooled cells were resuspended in 1X annexin-binding buffer. Cell density

was determined and the pooled cells diluted with 1X annexin-binding buffer to a concentration of 0.5 x 10^6 cells/mL. FITC annexin V (1 in 20 dilution) and propidium iodide (1 in 100 dilution) was added to the pooled cells which were then incubated at room temperature for 15 minutes. The stained cells were diluted in 1X annexin-binding buffer (1 in 5 dilution), mixed gently and kept on ice. Fluorescence emission at 530 nm (e.g., FL1) and >575 nm (e.g., FL3) was measured using flow cytometry. Populations separated into three groups: live cells showing only a low level of fluorescence, apoptotic cells showing green fluorescence and dead cells showing both red and green fluorescence.

2.9 Statistical Analysis

Statistical analysis was performed using software embedded in Prism 5 for Mac OS X Version 5.0c (GraphPad Software Inc, La Jolla, CA). Data were summarised as the mean and standard error, or the median and range as appropriate. Data were compared following testing for deviance from a Gaussian distribution and for equality of variance using parametric analysis (parametric one-way analysis of variance, paired/unpaired variants of student's t-test, linear regression) or non-parametric analysis (Mann-Whitney U test, Wilcoxon matched pairs test, Spearman's rank correlation, Kruskal–Wallis one-way analysis of variance) as appropriate. Statistical significance was taken as $p<0.05$.

Chapter 3: Primary bronchial fibroblast cells *in vitro* experiments

3 Bronchial fibroblast cells *in vitro* experiments

3.1 Introduction

The pathogenesis of asthma is characterised by Th2 type inflammation and remodelling, which leads to bronchial hyper-responsiveness, increased mucus production and airway narrowing. The consensus of opinion is that this process is primarily driven by an inappropriate Th2 inflammatory response to inhaled aeroallergens that might be further modified by the surrounding structural cell (Martinez and Vercelli, 2013).

3.1.1 Role of bronchial fibroblast cells in asthma

A key role of fibroblasts in the airways is to synthesize extracellular matrix. They are also involved in wound healing and in particular in synthesis of growth factors. Thickening of the sub-mucosal basement membrane, which is a characteristic of asthma, is caused by the deposition of collagen types III and V, laminin, fibronectin and tenascin by bronchial fibroblasts (Roche et al., 1989, Li and Wilson, 1997, Laitinen et al., 1997, Hoshino et al., 1998a, Saotome et al., 2003, Batra et al., 2003, Degen et al., 2009, Pegorier et al., 2010). The increased airways mucosal vascularity also observed in asthma is likely caused by increased expression of VEGF and related factors in the airways. These factors may arise from various sources, including fibroblasts, endothelial cells, airway smooth muscle cells and bronchial epithelial cells (Psarras et al., 2006, Capetandes et al., 2007, Simcock et al., 2008, Corrigan et al., 2011). Bronchial fibroblasts are also able directly to modulate the immune response by releasing a variety of inflammatory cytokines and chemokines (i.e. IL-6, IL-8, eotaxin and RANTES) (Teran et al., 1999, Le Bellego et al., 2009).

3.1.2 Effects of cigarette smoke exposure on bronchial fibroblast cells function

On exposure to cigarette smoke bronchial fibroblasts release a variety of pro-inflammatory and pro-remodelling cytokines. The pro-inflammatory cytokines and chemokines released by bronchial fibroblast on cigarette smoke exposure include IL-8, granulocyte-macrophage colony-stimulating factor/CSF2, granulocyte colony-stimulating factor/CSF3 and monocyte chemotactic protein-1/CCL2 (Sato et al., 1999, Martey et al., 2004, Moretto et al., 2009). The remodelling cytokines known to be

released on cigarette smoke exposure include the matrix metalloproteinases (MMP)-1 and MMP-2 (Kim et al., 2004, Ning et al., 2007). Bronchial fibroblasts therefore have the potential to affect not only the inflammatory response but also airways remodelling seen in smoking asthmatics.

3.1.3 Summary

Smoking causes a reduction in symptom control, acceleration of the decline in lung function and increased exacerbations in asthmatic patients (Althuis et al., 1999, James et al., 2005, Boulet et al., 2006, Eisner and Iribarren, 2007, Chaudhuri et al., 2008). Despite the clinical effects of smoking on asthma, there have been surprisingly few reports about its effects on inflammation and remodelling. Published studies to date have found no differences in the degree of airways remodelling, other than epithelial remodelling, in smoking asthmatics compared to non-smoking asthmatics. An increase in the neutrophil count in the bronchial mucosa of smoking asthmatic patients was also demonstrated (St-Laurent et al., 2008, Broekema et al., 2009). The interplay between structural cells and inflammatory cells in asthma will likely lead us to answers as to the reason why smoking causes a poorer outcome in asthma patients.

An important unresolved question is the role bronchial fibroblast plays in airway remodelling and inflammation in smoking asthmatic patients. The objective of this chapter is to investigate the role that fibroblast may play in the induction and maintenance of the hypothesized IL-17A mediated neutrophilic inflammation induced by cigarette smoking in asthma. Another objective of this chapter is to investigate the role that the fibroblast may play in the hypothesized increased angiogenesis induced by cigarette smoking in asthma. These hypotheses were investigated by performing in vivo experiments on primary bronchial fibroblast with the aim of addressing the following hypotheses:

Neutrophilia hypothesis

1. Cigarette smoke extract stimulates bronchial fibroblasts to produce IL-6 and Transforming Growth Factor-β1 (TGF-β1).
2. Cigarette smoke extract is able to enhance production of the pro-inflammatory cytokines produced by IL-17A stimulation of bronchial fibroblasts.
3. Bacterial and viral infections are potentially able to enhance the expression of the pro-inflammatory cytokines induced by cigarette smoke extract on bronchial fibroblasts.
4. These effects are mediated by distinct signal transduction pathways.

Angiogenesis hypothesis

1. Cigarette smoke extract stimulates bronchial fibroblasts to produce angiogenic growth factors (vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), epithelial growth factor (EGF) and platelet derived growth factor (PDGF)).
2. Cigarette smoke extract is able to enhance the production of angiogenic growth factors produced by IL-17A stimulation of bronchial fibroblasts.
3. Bacterial and viral infections are potentially able to enhance the expression of angiogenic growth factors induced by cigarette smoke extract on bronchial fibroblasts.
4. These effects are mediated by distinct signal transduction pathways.

3.2 Effects of cigarette smoke extract on bronchial fibroblast cells

Primary bronchial fibroblasts were isolated from normal non-smoking, non-asthmatic adult donors. Experiments were conducted on cells from Passage 4 and 5, at 100% confluence, in 12 well tissue culture plates as described in section 2.3.1 above. Following 24, 48 and 72 hours of culture, the supernatants were harvested and stored at -80^{o}C. VEGF, IL-6, bFGF, PDGF and TGF-β1 were measured using ELISA as described in section 2.5.

3.2.1 Fibroblast viability

In order to ensure that the results obtained from our in vivo experiments were due to the direct stimulation of primary bronchial fibroblasts by CSE and not the result of any cytotoxic effect of CSE, we initially assessed the viability of primary bronchial fibroblasts across a concentration range of CSE over a culture period of 24, 48 and 72 hours.

Primary bronchial fibroblasts were cultured in 12 well culture plates and exposed to a concentration series of CSE as described in section 2.3.1.1. The supernatants were removed and viability was assessed using trypan blue staining of the adherent cells and percentage confluence was estimated. Using trypan blue exclusion, the viability at least of adherent cells was maintained on exposure to CSE at up to 5% for up to 72 hours, the entire time course of all the experiments. With CSE concentrations in excess of 5%, the cells were less confluent, which could have reflected death and detachment of cells even though adherent cells remained viable (Figure 5).

Annexin V and propidium iodide (PI) viability measurement was compromised by the tendency of non-adherent cells to clump. Nevertheless the data appeared to confirm that the viability of single cells was maintained in concentrations of CSE up to 5%, at least for 24 hours of culture (Figure 6 and Figure 7).

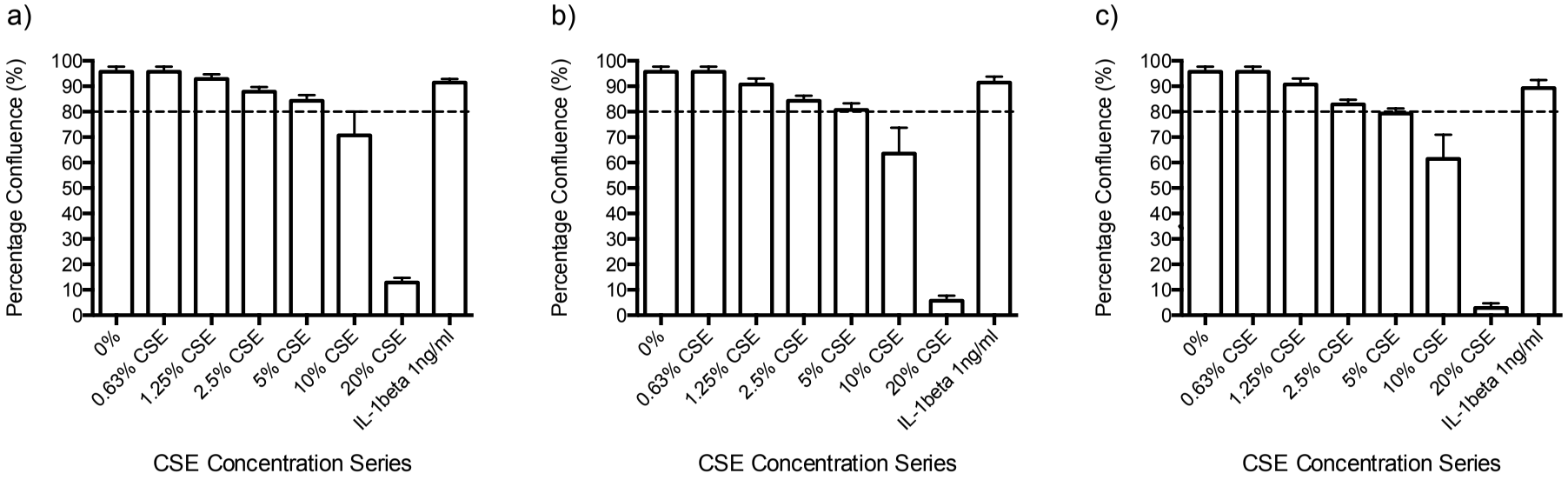

Figure 5: Primary fibroblast viability assessed by trypan blue exclusion

Primary bronchial fibroblasts were cultured in 12 well tissue culture plates and exposed to a concentration series of CSE as described in section 2.3.1.1. Viability and percentage confluence were measured by trypan blue exclusion at a) 24 hours, b) 48 hours and c) 72 hours. Percentage confluence and viability was maintained above 80% at all time points with concentrations below 5% CSE. At concentrations of 10% or above there was a significant reduction in cell viability and percentage confluence. Mean ± SD, n = 7 .

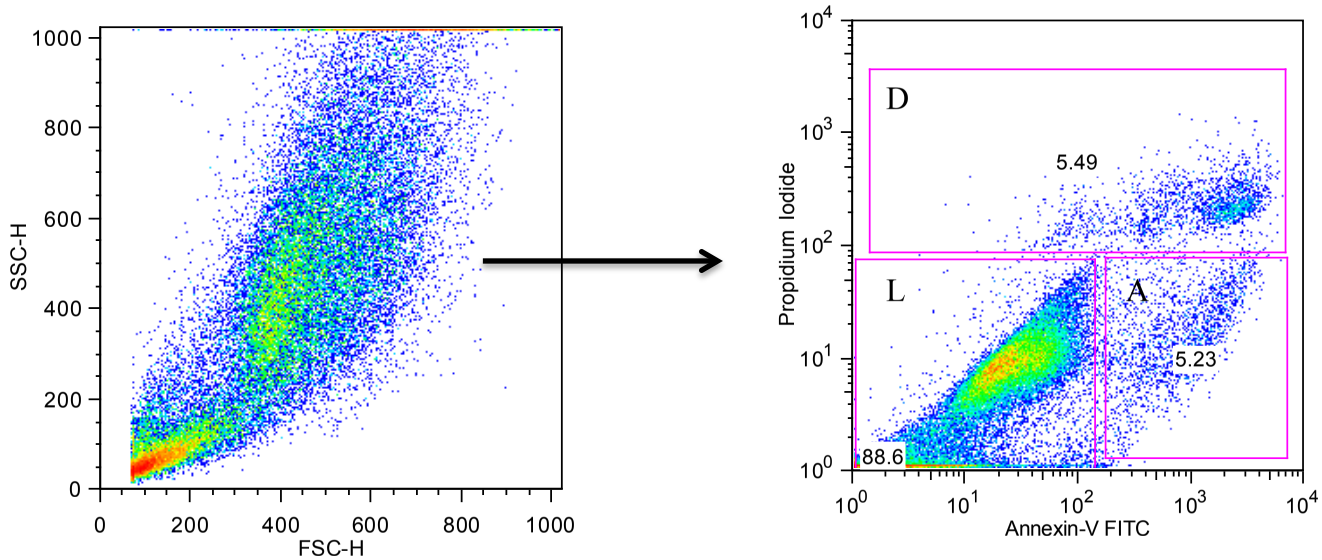

Figure 6: Flow cytometry gating strategy for fibroblast propidium iodide and annexin-V FITC staining

Primary bronchial fibroblasts were cultured in 12 well tissue culture plates and exposed to a concentration series of CSE. The supernatants were harvested and the adherent cells were detached. Annexin-V FITC and propidium iodide staining was performed on the cells in the supernatant and detached adherent cells. Flow cytometric analysis was performed and the above gating strategy was used. L = Live, A = Apoptotic, D = Dead.

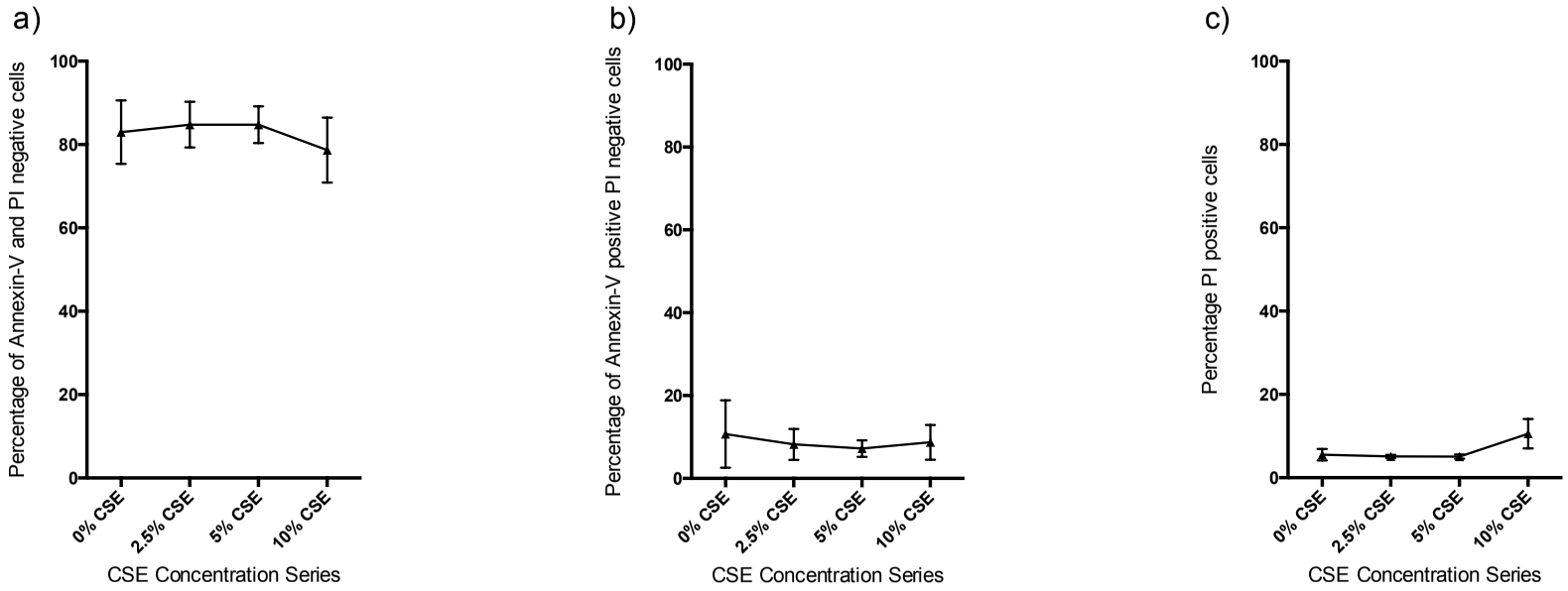

Figure 7: Primary fibroblast viability assessed using propidium iodide and annexin-V

Primary bronchial Ffibroblasts were cultured in 12 well tissue culture plates and exposed to a concentration series of CSE. Viability was assessed using propidium iodide (PI) and annexin-V FITC staining and flow cytometric analysis. a) Percentages of live cells at 24 hours of culture were - Baseline 82.97% ± 7.601, 2.5% CSE 84.77% ± 5.472, 5% CSE 84.77% ± 4.387, and 10% CSE 78.67 ± 7.778. b) Percentages of apoptotic cells at 24 hours of culture were - Baseline 10.71% ± 8.138, 2.5% CSE 8.217% ± 3.732, 5% CSE 7.200% ± 1.977, and 10% CSE 8.717% ± 4.182. c) Percentages of dead cells at 24 hours of culture were - Baseline 5.507% ± 1.379, 2.5% CSE 5.103 ± 0.3953, 5% CSE 5.067% ± 0.5103, and 10% CSE 10.56% ± 3.519. Mean ± SD, n = 3.

3.2.2 Vascular endothelial growth factor expression

Primary bronchial fibroblasts were cultured in 12 well tissue culture plates and exposed to a concentration series of CSE. Vascular Endothelial Growth Factor (VEGF) release by the cells was measured using ELISA after 24 hours, 48 hours and 72 hours of culture. CSE induced VEGF release in a concentration-dependent fashion after 24 hours, 48 hours and 72 hours of culture (Anova $p < 0.0001$, $p = 0.0157$ and $p < 0.0001$ respectively). CSE exposure further increased VEGF release at all time points at concentrations which preserved fibroblast viability. IL-1β stimulation was used as a positive control (Asano-Kato et al., 2005). At 24 hours the maximum increase of VEGF was 2.257 ± 0.7711 fold above baseline, while that at 48 hours 2.253 ± 1.349 fold above baseline and at 72 hours 2.717 ± 1.006 fold above baseline (Mean ± SD) (Figure 8 and Table 1).

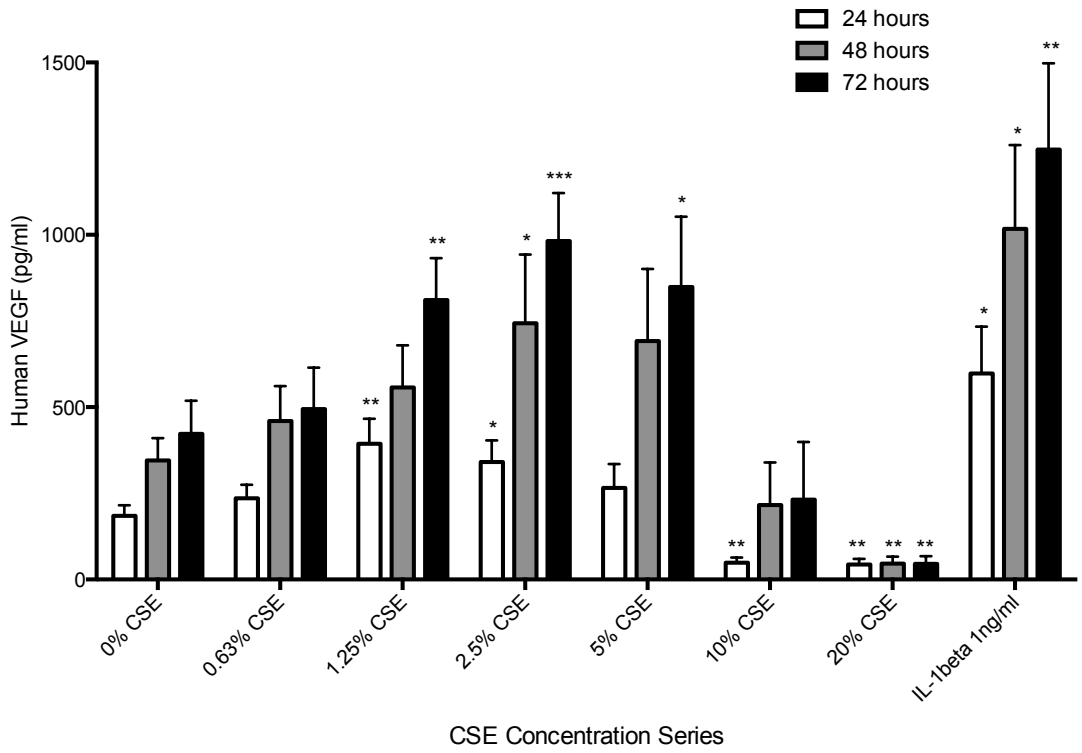

Figure 8: Effect of CSE on the time course of spontaneous VEGF production by primary human bronchial fibroblasts

Primary bronchial fibroblasts were cultured in 12 well tissue culture plates as described in section 2.3.1.1 and exposed to a concentration series of CSE. When stimulated with CSE, VEGF was released in concentration dependent manner at 24 hours, 48 hours and 72 hours of culture (Anova $p < 0.0001$, $p = 0.0157$, and $p < 0.0001$ respectively). Paired t-test * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Mean ± SEM, n = 7

Time Course (hours)	Baseline	Concentration of Cigarette Smoke Extract						IL-1β 1 ng/ml	Anova p value
		0.63%	1.25%	2.5%	5%	10%	20%		
24	184.3 ± 31.29	235.8 ± 39.15	393.9 ± 72.12 §	340.9 ± 62.82 †	266.0 ± 69.10	48.39 ± 15.54 §	43.15 ± 16.84 §	597.9 ± 135.6 †	< 0.0001
48	345.3 ± 64.60	459.8 ± 101.1	557.4 ± 121.9	743.3 ± 199.7 †	691.6 ± 209.6	215.8 ± 123.8	46.17 ± 20.31 §	1017 ± 242.8 †	0.0157
72	422.7 ± 95.80	494.8 ± 119.8	811.0 ± 121.1 §	981.8 ± 139.5 ‡	848.5 ± 204.2 †	231.8 ± 167.3	45.28 ± 22.10 §	1248 ± 249.9 §	< 0.0001

VEGF pg/ml Mean ± SEM, n = 7; Paired t-test † $p < 0.05$, § $p < 0.01$, ‡ $p < 0.001$ vs baseline

Anova comparing concentrations from baseline to 5% CSE

Table 1: Effect of CSE on the time course of spontaneous VEGF production by primary human bronchial fibroblasts

3.2.3 Interleukin-6 expression

Primary bronchial fibroblasts were cultured in 12 well tissue culture plates and exposed to a concentration series of CSE. Interleukin-6 (IL-6) release by the cells was measured using ELISA after 24 hours, 48 hours and 72 hours of culture. CSE induced IL-6 release in a concentration-dependent fashion after 24 hours, 48 hours and 72 hours of culture (Anova $p = 0.0002$, $p = 0.0042$ and $p < 0.0001$ respectively). CSE exposure further increased IL-6 release at all time points at concentrations which preserved fibroblast viability. IL-1β stimulation was used as a positive control (Chen et al., 2005). At 24 hours the maximum increase of IL-6 was 4.682 ± 4.775 fold above baseline, while that at 48 hours 2.663 ± 1.152 fold above baseline and at 72 hours 4.202 ± 3.625 fold above baseline (Mean ± SD) (Figure 9 and Table 2).

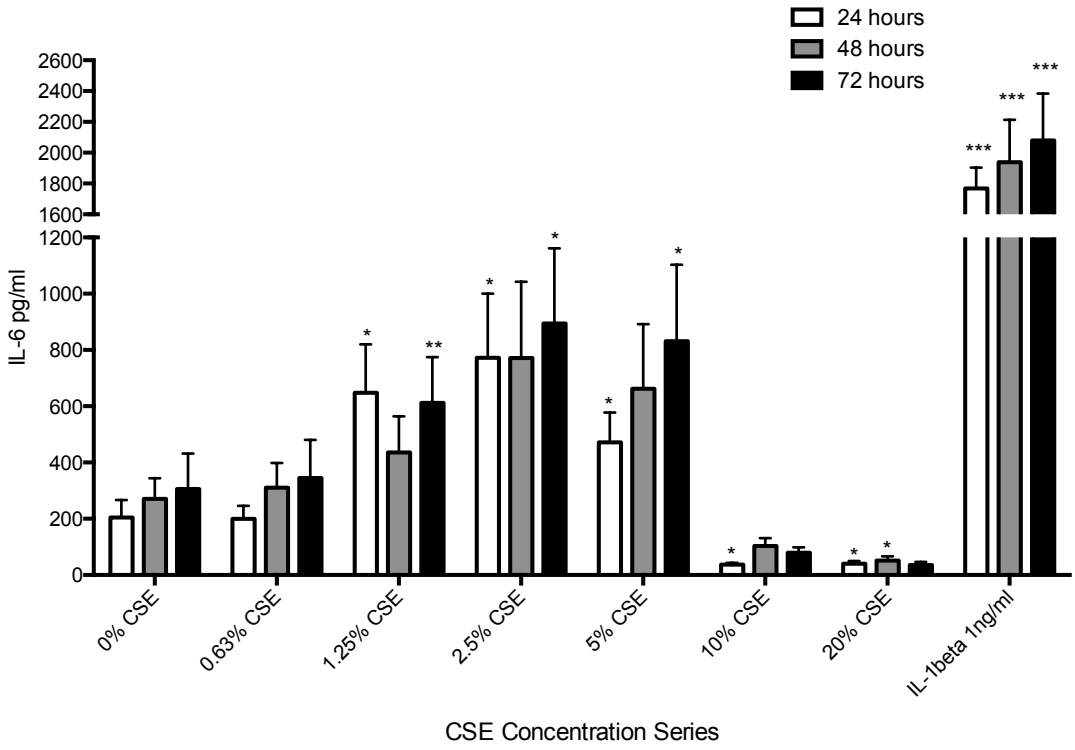

Figure 9: Effect of CSE on the time course of spontaneous IL-6 production by primary human bronchial fibroblasts

Primary bronchial fibroblasts were cultured in 12 well tissue culture plates as described in section 2.3.1.1 and exposed to a concentration series of CSE. When stimulated with CSE, IL-6 was released in a concentration dependent manner at 24 hours, 48 hours and 72 hours of culture (Anova $p = 0.0002$, $p = 0.0042$ and $p < 0.0001$ respectively). Paired t-test * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Mean ± SEM, n = 7

Time Course (hours)	Concentration of Cigarette Smoke Extract							IL-1β 1 ng/ml	Anova p value
	Baseline	0.63%	1.25%	2.5%	5%	10%	20%		
24	204.6 ± 61.88	200.0 ± 46.32	647.7 ± 172.0 †	772.2 ± 228.3 †	471.7 ± 105.8 †	36.77 ± 6.919 †	40.12 ± 9.449 †	1768 ± 136.2 ‡	0.0002
48	270.7 ± 73.63	310.3 ± 87.73	435.9 ± 128.5	771.5 ± 271.4	662.6 ± 229.6	102.9 ± 27.89	51.13 ± 15.75 †	1938 ± 274.6 ‡	0.0042
72	305.4 ± 126.4	344.5 ± 135.9	611.7 ± 163.1§	894.4 ± 267.2 †	831.0 ± 272.0 †	79.17 ± 19.23	35.78 ± 10.50	2080 ± 304.0 ‡	< 0.0001

IL-6 pg/ml Mean ± SEM, n = 7; Paired t-test † $p < 0.05$, § $p < 0.01$, ‡ $p < 0.001$ vs baseline

Anova comparing concentrations from baseline to 5% CSE

Table 2: Effect of CSE on the time course of spontaneous IL-6 production by primary human bronchial fibroblasts

3.2.4 Transforming growth factor-beta 1 expression

Primary bronchial fibroblasts were cultured in 12 well tissue culture plates and exposed to a concentration series of CSE. Transforming Growth Factor-β1 (TGF-β1) release was measured using ELISA at 24 hours, 48 hours and 72 hours of culture. CSE induced TGF-β1 release by primary bronchial fibroblasts after 72 hours of culture (Anova $p = 0.0050$). At concentrations of CSE previously shown to preserve the viability of the fibroblasts, spontaneous release of TGF-β1 was significantly increased at the 72 hours time point, but not at earlier time points. At 72 hours the maximum increase of TGF-β1 was 2.645 ± 3.496 fold above baseline (Mean ± SD) (Figure 10 and Table 3).

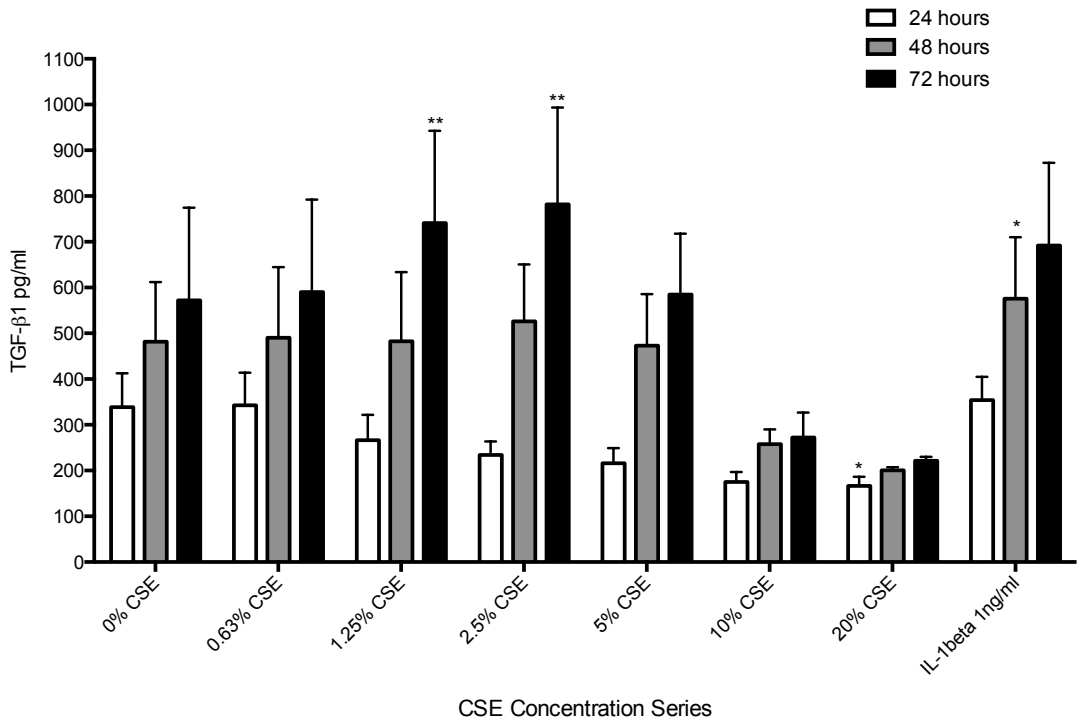

Figure 10: Effect of CSE on the time course of spontaneous TGF-β1 production by primary human bronchial fibroblasts

Primary bronchial fibroblasts were cultured in 12 well tissue culture plates as described in section 2.3.1.1 and exposed to a concentration series of CSE. When stimulated with CSE, TGF-β1 was released in a concentration dependent manner at 72 hours of culture (Anova p = 0.0050). Paired t-test * $p < 0.05$, ** $p < 0.01$, Mean ± SEM, n = 7

Time Course (hours)	Concentration of Cigarette Smoke Extract							IL-1β 1 ng/ml	Anova p value
	Baseline	0.63%	1.25%	2.5%	5%	10%	20%		
24	338.4 ± 74.10	342.6 ± 71.55	266.4 ± 55.53	234.1 ± 29.76	215.9 ± 33.08	175.2 ± 21.62	166.5 ± 20.09 †	354.2 ± 50.58	0.1104
48	481.5 ± 130.5	490.4 ± 154.3	482.6 ± 151.4	526.1 ± 124.4	472.9 ± 112.6	258.0 ± 31.99	200.4 ± 7.083	575.7 ± 134.6 †	0.9396
72	571.8 ± 202.9	590.4 ± 202.1	740.8 ± 201.9 §	781.9 ± 211.5 §	584.7 ± 133.3 †	272.4 ± 54.39	221.3 ± 8.778	691.9 ± 180.9	0.0050

TGF-β1 pg/ml Mean ± SEM, n = 7; Paired t-test † $p < 0.05$, § $p < 0.01$ vs baseline

Anova comparing concentrations from baseline to 5% CSE

Table 3: Effect of CSE on the time course of spontaneous TGF-β1 production by primary human bronchial fibroblasts

3.2.5 Basic fibroblast growth factor expression

Primary bronchial fibroblasts were cultured in 12 well tissue culture plates and exposed to a concentration series of CSE. Basic Fibroblast Growth Factor (bFGF) release was measured using ELISA at 24hrs, 48hrs and 72hrs of culture. CSE induced bFGF release in a concentration-dependent fashion after 24 hours, 48 hours and 72 hours of culture, (Anova $p < 0.0001$, $p < 0.0001$ and $p < 0.0001$ respectively). Spontaneous release of bFGF by the cultured cells was undetectable up to 72 hours. CSE exposure elevated bFGF expression in a concentration-dependent fashion most clearly following 24 hours of exposure. This effect was reduced after longer periods of exposure (48 and 72 hours) and most marked at concentrations of CSE which may have compromised the viability of the cells. Interestingly the IL-1β positive control elicited a similar temporal pattern of bFGF induction. The maximal increase of bFGF at 24 hours was 474.1pg/ml ± 57.39, 48 hours was 179.0pg/ml ± 23.81 and 72 hours was 160.6pg/ml ± 45.31 (Mean ± SEM) (Figure 11 and Table 4).

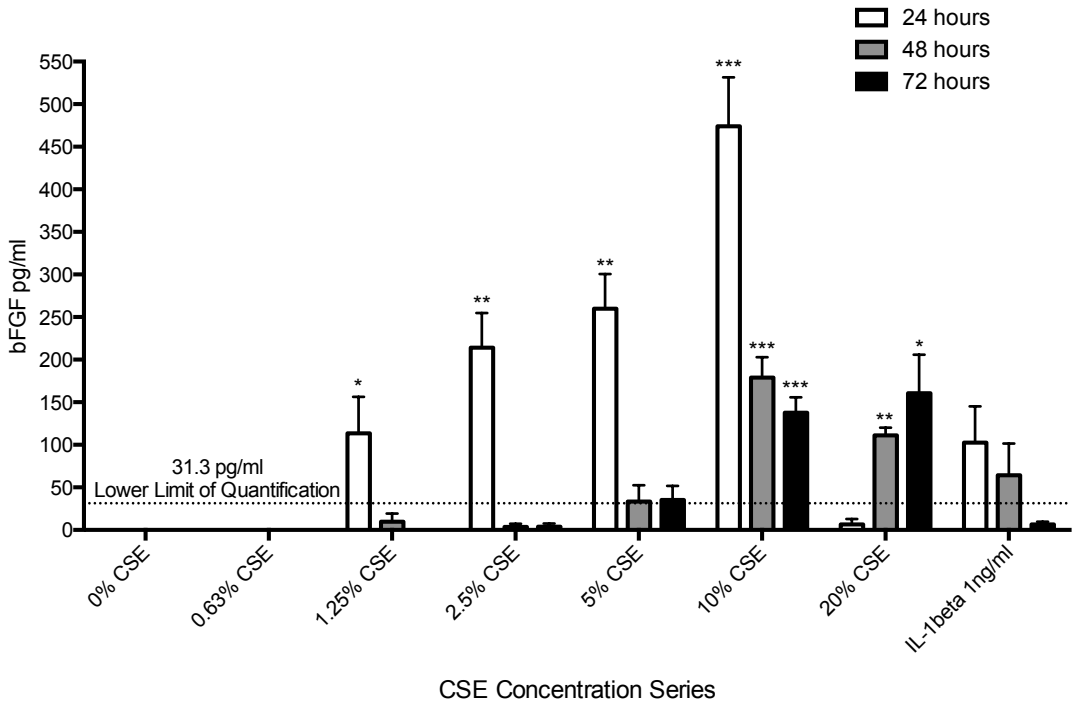

Figure 11: Effect of CSE on the time course of spontaneous bFGF production by primary human bronchial fibroblasts

Primary bronchial fibroblasts were cultured in 12 well tissue culture plates as described in section 2.3.1.1 and exposed to a concentration series of CSE. When stimulated with CSE, bFGF was released in a concentration-dependent manner at 24 hours, 48 hours and 72 hours of culture (Anova $p < 0.0001$, $p < 0.0001$ and $p < 0.0001$ respectively). Paired t-test * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Mean ± SEM, n = 6

Time Course (hours)	Baseline	Concentration of Cigarette Smoke Extract						IL-1β 1 ng/ml	Anova p value
		0.63%	1.25%	2.5%	5%	10%	20%		
24	0 ± 0	0 ± 0	113.6 ± 42.81 †	214.0 ± 40.75 §	259.9 ± 40.50 §	474.1 ± 57.39 ‡	6.408 ± 6.408	102.7 ± 42.46	$p < 0.0001$
48	0 ± 0	0 ± 0	9.665 ± 9.665	3.660 ± 3.660	33.42 ± 19.14	179.0 ± 23.81 ‡	111.0 ± 9.190 §	64.19 ± 37.33	$p < 0.0001$
72	0 ± 0	0 ± 0	0 ± 0	3.750 ± 3.750	35.26 ± 16.54	137.8 ± 18.06 ‡	160.6 ± 45.31 †	6.533 ± 3.171	$p < 0.0001$

bFGF pg/ml Mean ± SEM, n = 6; Paired t-test † $p < 0.05$, § $p < 0.01$, ‡ $p < 0.001$ vs baseline;

Anova comparing concentrations from baseline to 10% CSE (24 hours) and to 20% (48 and 72 hours)

Table 4: Effect of CSE on the time course of spontaneous bFGF production by primary human bronchial fibroblasts

3.2.6 Expression of epithelial growth factor and platelet derived growth factor BB

Primary bronchial fibroblasts failed to release detectable epithelial growth factor (EGF) or platelet derived growth factor BB (PDGF-BB) after up to 72 hours of culture either spontaneously or in the presence of a concentration series of CSE as in previous experiments (data not shown).

3.3 Effect on dexamethasone on cigarette smoke extract induced expression of pro-inflammatory and remodelling cytokines

The concentration time course experiment for VEGF, IL-6 and TGF-β1 (as described in section 3.2) demonstrated that 2.5% CSE was the optimal concentration of CSE at which fibroblast viability was not affected and a near maximal response to CSE was observed for the release of these cytokines. In addition, the optimal culture time for VEGF and IL-6 expression was 24 hours while that for TGF-β1 expression was 72 hours. Following the concentration time course experiments, supernatants were harvested at 24 hours for VEGF and IL-6 expression and 72 hours for TGF-β1 expression

We investigated whether corticosteroids are able to inhibit the pro-inflammatory and remodelling cytokines released when primary bronchial fibroblasts are exposed to cigarette smoke extract. Primary bronchial fibroblasts were cultured in 12 well tissue culture plates and exposed to 2.5% CSE and a concentration series of dexamethasone (10^{-6} M, 10^{-7} M, and 10^{-8} M) for 24 hours and 72 hours of culture. Dexamethasone used was reconstituted in absolute ethanol and diluted using medium. At a concentration of ethanol equivalent to that used to dilute the highest concentration of ethanol (10^{-6} M) was used as vehicle control. Supernatants were harvested at 24 hours and VEGF and IL-6 expression measured using ELISA. Supernatants were also harvested at 72 hours and TGF-β1 expression measured using ELISA.

Dexamethasone at 10^{-6} M and 10^{-7} M significantly inhibited the expression of VEGF induced by 2.5% CSE (2.5% CSE 291.2 pg/ml ± 73.41; 2.5% CSE with Dex 10^{-6} M 133.1 pg/ml ± 41.24, p = 0.0086; 2.5% CSE with Dex 10^{-7} M 136.5 pg/ml ± 34.90, p = 0.0130). Dexamethasone also tended to suppress spontaneous expression of VEGF (Baseline 108.9 pg/ml ± 36.13; Dex 10^{-6} M 68.65 pg/ml ± 20.65, p = 0.0772; Dex 10^{-7} M 75.15 pg/ml ± 22.09, p = 0.1192; Dex 10^{-8} M 57.57 pg/ml ± 19.58, p = 0.0538). Vehicle control did not affect spontaneous expression of VEGF (100.9 pg/ml ± 37.66, p = 0.1471) (Figure 12 a and Table 5).

Dexamethasone at 10^{-6}, 10^{-7} and 10^{-8} M also significantly inhibited the expression of IL-6 induced by 2.5% CSE (2.5% CSE 231.5 pg/ml ± 49.85; 2.5% CSE with Dex 10^{-6} M 76.24 pg/ml ± 20.87, p = 0.0104; 2.5% CSE with Dex 10^{-7} M 135.6 pg/ml ± 49.63, p = 0.0174; 2.5% CSE with Dex 10^{-8} M 104.8 pg/ml ± 40.10, p = 0.0378) and also tended to suppress spontaneous expression of IL-6 (Baseline 96.49 pg/ml ± 24.11; Dex 10^{-6} M 65.81 pg/ml ± 16.15, p = 0.3472; Dex 10^{-7} M 80.99 pg/ml ± 21.17, p = 0.5080; Dex 10^{-8} M 58.48 pg/ml ± 25.87, p = 0.0587). Vehicle control did not affect spontaneous expression of IL-6 (69.53 pg/ml ± 18.81, p = 0.0654) (Figure 12 b and Table 5).

Finally, dexamethasone at 10^{-6}, 10^{-7} and 10^{-8} M also significantly inhibited TGF-β1 expression induced by 2.5% CSE (2.5% CSE 568.5 pg/ml ± 81.19; 2.5% CSE with Dex 10^{-6} M 484.5 pg/ml ± 97.20, p = 0.0425; 2.5% CSE with Dex 10^{-7} M 466.7 pg/ml ± 97.20, p = 0.0440; 2.5% CSE with Dex 10^{-8} M 466.7 pg/ml ± 97.09, p = 0.0176). There was no effect on spontaneous expression of TGF- β1 (Baseline 458.8 pg/ml ± 188.5; Dex 10^{-6} M 482.9 pg/ml ± 134.1, p = 0.6882; Dex 10^{-7} M 442.8 pg/ml ± 127.8, p = 0.7747; Dex 10^{-8} M 384.1 pg/ml ± 66.28, p = 0.1319). Vehicle control did not affect spontaneous expression of TGF-β1 (483.1 pg/ml ± 95.73, p = 0.1572) (Figure 12 c and Table 5).

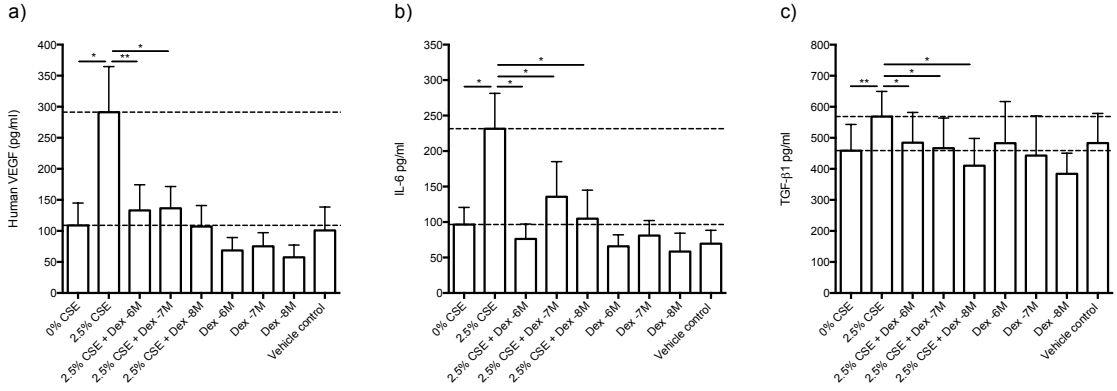

Figure 12: Dexamethasone suppression of pro-inflammatory and remodelling cytokines induced by cigarette smoke in primary bronchial fibroblasts

Primary bronchial fibroblasts were cultured in 12 well tissue culture plates as described in section 2.3.1.2 and exposed to 2.5% CSE and a concentration series of dexamethasone (10^{-6} M, 10^{-7} M, and 10^{-8} M) for 24 hours and 72 hours of culture. a) 2.5% CSE induced the expression of VEGF and this was inhibited by dexamethasone. b) 2.5% CSE induced the expression of IL-6 and this was inhibited by dexamethasone. c) 2.5% CSE induced the expression of TGF-β1 and this was inhibited by dexamethasone. Paired t-test * $p < 0.05$, ** $p < 0.01$, Mean ± SEM, n = 5

	Baseline	2.5% CSE	2.5% CSE with Dex 10^{-6} M	2.5% CSE with Dex 10^{-7} M	2.5% CSE with Dex 10^{-8} M
VEGF	108.9 pg/ml ± 36.13	291.2 pg/ml ± 73.41 †	133.1 pg/ml ± 41.24 §§	136.5 pg/ml ± 34.90 §	107.0 pg/ml ± 33.93
IL-6	96.49 pg/ml ± 24.11	231.5 pg/ml ± 49.85 †	76.24 pg/ml ± 20.87 §	135.6 pg/ml ± 49.63 §	104.8 pg/ml ± 40.10 §
TGF-β1	458.8 pg/ml ± 188.5	568.5 pg/ml ± 81.19 ††	484.5 pg/ml ± 97.20 §	466.7 pg/ml ± 97.20 §	466.7 pg/ml ± 97.09 §

Mean ± SEM, n = 5;

Paired t-test † $p < 0.05$, †† $p < 0.01$, vs baseline;

Paired t-test § $p < 0.05$, §§ $p < 0.01$ vs 2.5% CSE

Table 5: Dexamethasone suppression of pro-inflammatory and remodelling cytokines induced by cigarette smoke in primary bronchial fibroblasts

3.4 Modification of cigarette smoke extract induced expression of pro-inflammatory and remodelling cytokines by surrogates of bacterial and viral induced inflammation

Following on from this we wished to investigate possible influences of surrogates of local bacterial and viral infection on the release of pro-inflammatory and remodelling cytokines by primary bronchial fibroblasts exposed to cigarette smoke extract.

Lipoteichoic Acid (LTA) is a major constituent of the cell wall of Gram positive bacteria. It is a ligand for Toll-like Receptor 2 (TLR2) and we used it as a surrogate for bacterial infections. The effect of LTA on the expression of VEGF, IL-6 and TGF-β1 by primary bronchial fibroblasts was investigated in order to i) identify if LTA is able to stimulate the release of these cytokines and ii) determine a concentration range of LTA that is the minimal biologically active concentration.

Primary bronchial fibroblasts were cultured in 12 well tissue culture plates and exposed to a concentration series of LTA. A concentration of 10 μg/ml of LTA has been shown to be effective at stimulating the release of IL-6 by dermal fibroblasts (Perfetto et al., 2003). We thus inferred that the minimal biologically active concentration of LTA is likely to be below 10 μg/ml. We investigated a concentration series that consisted of both halving dilution and 10 fold dilutions below 10 μg/ml of LTA with the lowest concentration investigated a 100 fold dilution of 10 μg/ml (i.e. 0.1, 0.5, 1, and 5 μg/ml). LTA in the concentration range used evoked no significant variation in the spontaneous expression of VEGF, IL-6 and TGF-β1 (Anova $p = 0.2303$, $p = 0.2710$ and $p = 0.2484$ respectively). There however appeared to be a trend for a concentration dependent increase in spontaneous expression of VEGF and IL-6, while no trend was seen on spontaneous expression of TGF-β1, in the concentration range of LTA used (Figure 13).

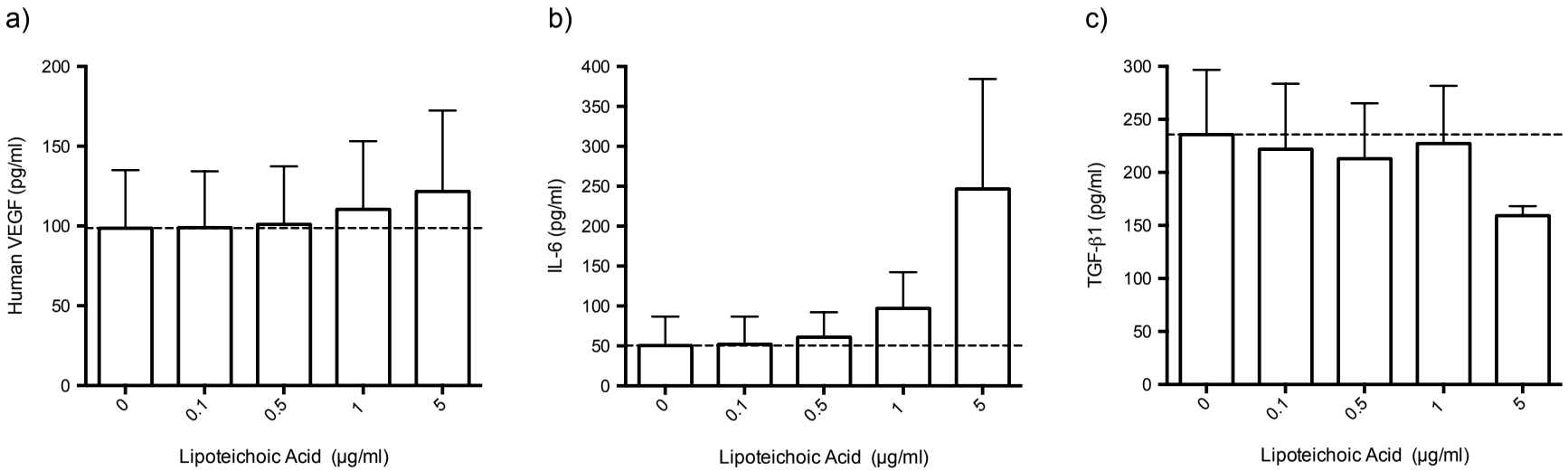

Figure 13: Response of primary bronchial fibroblasts to lipoteichoic acid concentration series

Primary bronchial fibroblasts were cultured in 12 well tissue culture plates as described in section 2.3.1.3 and exposed to a concentration series of LTA (0 – 5 µg/ml). a) A trend for a concentration dependent increase in spontaneous expression of VEGF is seen in this concentration range (Anova p = 0.2303). b) A trend for a concentration dependent increase in spontaneous expression of IL-6 is seen in this concentration range (Anova p = 0.2710) c) No effect on spontaneous expression of TGF-β1 was seen in this concentration range (Anova p = 0.2484). Mean ± SEM, n =4

Polyinosinic:polycytidylic acid (Poly I:C) is a synthetic analogue of double-stranded RNA (dsRNA). Group III viruses (Baltimore classification) are dsRNA viruses and dsRNA from these viruses are recognised by the host via Toll-like Receptor 3 (TLR3). The effect of Poly I:C on the expression of VEGF, IL-6 and TGF-β1 in primary bronchial fibroblasts was investigated in order to i) identify if Poly I:C is able to stimulate the release of these cytokines and ii) determine a concentration range of Poly I:C that is the minimal biologically active concentration.

Primary bronchial fibroblasts were cultured in 12 well tissue culture plates and exposed to a concentration series of Poly I:C. A concentration of 1 μg/ml of Poly I:C has been shown to be effective at stimulating the release of IL-6 by corneal fibroblasts (Liu et al., 2008). We thus inferred that the minimal biologically active concentration of Poly I:C is likely to be below 1 μg/ml. We investigated a concentration series that consisted of both halving dilution and 10 fold dilutions below 1 μg/ml of Poly I:C with the lowest concentration investigated a 100 fold dilution of 1 μg/ml (i.e. 0.01, 0.05, 0.1, and 0.5 μg/ml). Poly I:C in the concentration range used evoked no significant variation in the spontaneous expression of VEGF and TGF-β1 (Anova $p = 0.2623$ and $p = 0.5225$ respectively). There however appeared to be a trend for a concentration dependent increase in spontaneous expression of VEGF, while no trend was seen on spontaneous expression of TGF-β1, in the concentration range of Poly I:C used. Poly I:C in the concentration range used evoked a significant variation in the spontaneous production of IL-6 (Anova $p = 0.0135$) (Figure 14).

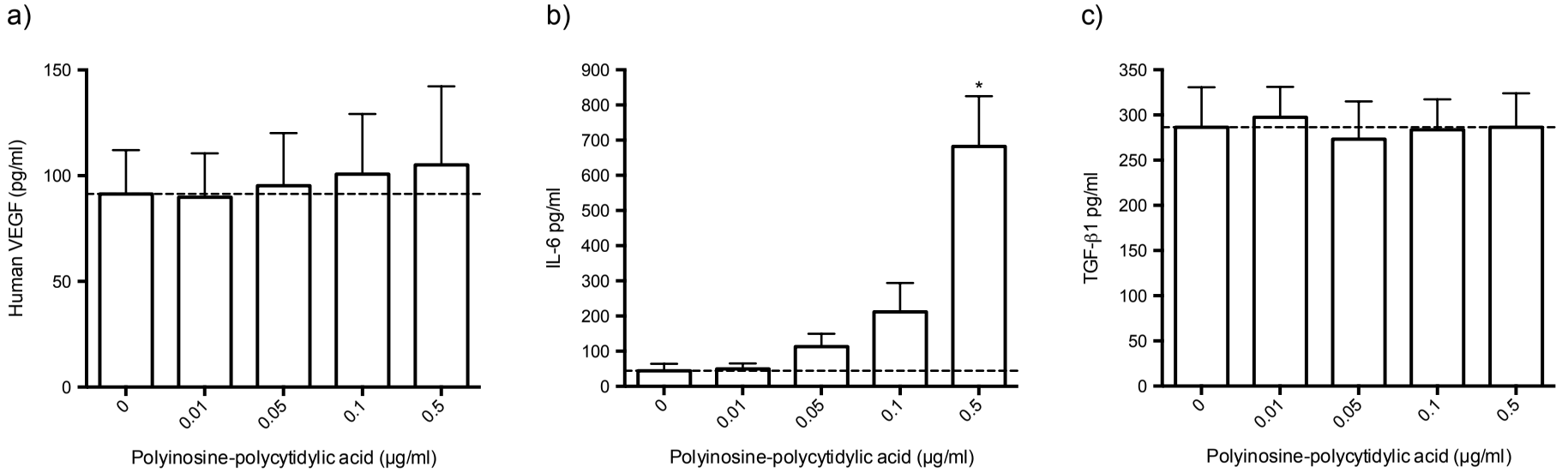

Figure 14: Response of primary bronchial fibroblasts to polyinosinic:polycytidylic acid concentration series

Primary bronchial fibroblasts were cultured in 12 well tissue culture plates as described in section 2.3.1.3 and exposed to a concentration series of Poly I:C (0 – 0.5 µg/ml). a) A trend for a concentration dependent increase in spontaneous expression of VEGF is seen in this concentration range (Anova $p = 0.2623$). b) A concentration dependent increase in spontaneous expression of IL-6 is seen in this concentration range (Anova $p = 0.0135$) c) No affect on spontaneous expression of TGF-β1 was seen in this concentration range (Anova $p = 0.5225$). Paired t-test * $p < 0.05$, Mean ± SEM, n =4

3.4.1 Lipoteichoic acid (LTA) - Toll-like receptor 2 agonist

In order provide some rationale for an interaction between the effects of exposure to cigarette smoke and bacterial infections of the respiratory tract we co-stimulated primary bronchial fibroblasts with cigarette smoke extract and LTA and measured the production of pro-inflammatory and remodelling cytokines. In view of the results described above (section 3.4) it was decided that we should use a concentration range of LTA from 0 – 1 μg/ml for these experiments. We had elected not to use a concentration of 5 μg/ml of LTA because our data showed evidence that this concentration of LTA has a high tendency to significantly increase the expression of IL-6. Primary bronchial fibroblasts were cultured in 12 well tissue culture plates and exposed to 0.1 μg/ml, 0.5 μg/ml and 1 μg/ml of LTA with or without the presence of 2.5% CSE. Supernatants were harvested at 24 hours and VEGF and IL-6 expression measured using ELISA. In view of the fact that LTA alone did not demonstrate a trend for a concentration-dependent increase in spontaneous expression of TGF-β1 (in the concentrations tested) we did not examine TGF-β1 expression.

As reported above, LTA alone in the concentration range 0 – 1 μg/ml did not significantly alter spontaneous production of VEGF by cultured primary bronchial fibroblasts. In the presence of 2.5% CSE, however, LTA at a concentration of 1 μg/ml significantly increased VEGF production ($p = 0.0499$) and there was evidence of a concentration related, synergistic response (Anova $p = 0.3093$) (Figure 15).

While LTA alone demonstrated a trend for a concentration dependent increase in IL-6 production by primary bronchial fibroblasts, the additional presence of 2.5% CSE further augmented IL-6 production but this effect was additive (Anova $p = 0.8377$) (Figure 16).

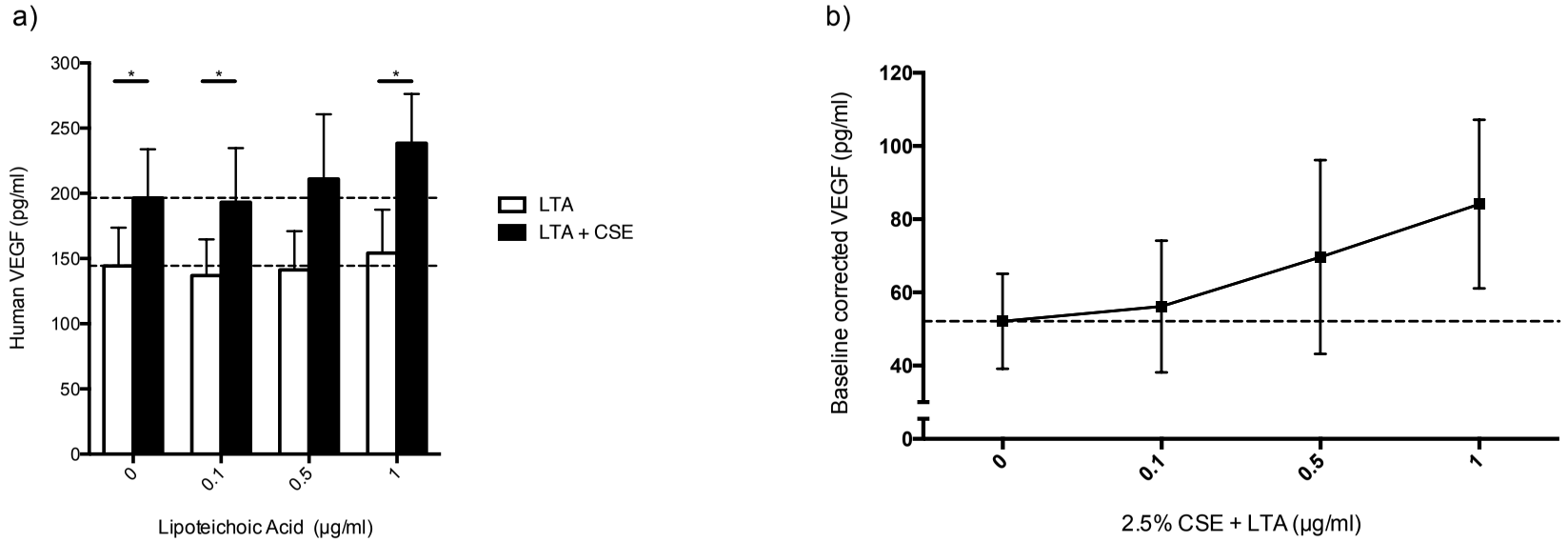

Figure 15: Co-stimulation of primary bronchial fibroblasts with lipoteichoic acid and cigarette smoke – VEGF

Primary bronchial fibroblasts were cultured in 12 well tissue culture plates as described in section 2.3.1.4 with a concentration series of LTA (0, 0.1, 0.5 and 1 µg/ml) with or without 2.5% CSE. a) CSE synergistically increased LTA-induced VEGF secretion (LTA without vs with CSE, 0 µg/ml 144.4 pg/ml ± 29.26 vs 196.5 ± 37.32, p = 0.0160; 0.1 µg/ml 137.0 pg/ml ± 27.76 vs 193.1 ± 41.52, p = 0.0355; 0.5 µg/ml 141.3 pg/ml ± 29.65 vs 211.0 ± 49.62, p = 0.0581; 1.0 µg/ml 154.3 pg/ml ± 33.15 vs 238.4 ± 37.92, p = 0.0217). b) Concentration-dependent effect trend of LTA on VEGF secretion in the presence of 2.5% CSE (Baseline 52.11 pg/ml ± 12.99; 0.1 µg/ml 56.17 pg/ml ± 18.00; 0.5 µg/ml 69.68 pg/ml ± 26.47; 1 µg/ml 84.12 pg/ml ± 23.03; Anova p = 0.3093). Paired t-test * $p < 0.05$, Mean ± SEM, n = 5

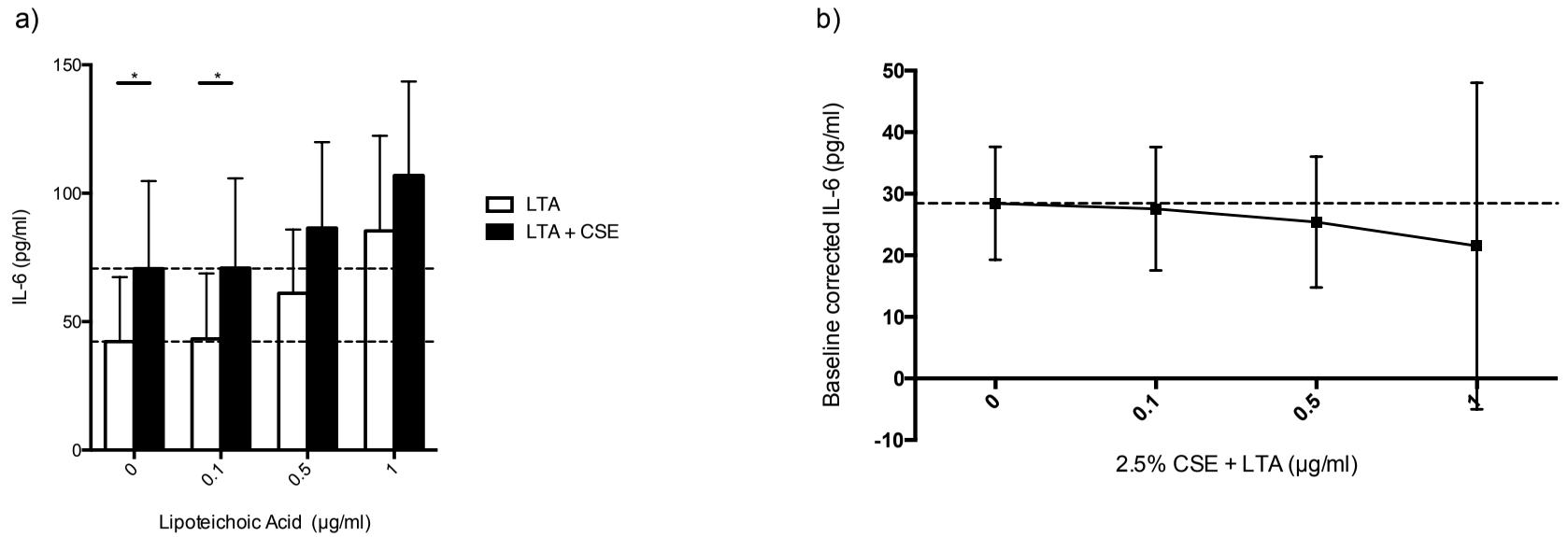

Figure 16: Co-stimulation of primary bronchial fibroblasts with lipoteichoic acid and cigarette smoke – IL-6

Primary bronchial fibroblasts were cultured in 12 well tissue culture plates as described in section 2.3.1.4 with a concentration series of LTA (0, 0.1, 0.5 and 1 µg/ml) with or without 2.5% CSE. a) CSE additively increased LTA-induced IL-6 secretion (LTA without vs with CSE, 0 µg/ml 42.22 pg/ml ± 25.13 vs 70.87 ± 34.14, p = 0.0211; 0.1 µg/ml 43.31 pg/ml ± 25.48 vs 70.87 ± 34.92, p = 0.0331; 0.5 µg/ml 61.02 pg/ml ± 24.82 vs 86.44 ± 33.43, p = 0.0537; 1.0 µg/ml 85.38 pg/ml ± 36.98 vs 106.9 ± 36.65, p = 0.4478). b) The magnitude of effect of 2.5% CSE on the expression of IL-6 was similar throughout the concentration range of LTA used (Baseline 28.44 pg/ml ± 9.175; 0.1 µg/ml 27.56 pg/ml ± 10.01; 0.5 µg/ml 25.41 pg/ml ± 10.62; 1 µg/ml 21.53 pg/ml ± 26.51; Anova p = 0.8377). Paired t-test * $p < 0.05$, Mean ± SEM, n = 5

3.4.2 Polyinosine-polycytidylic acid (Poly I:C) - Toll-like receptor 3 agonist

In order to provide some rationale for an interaction between the effects of exposure to cigarette smoke and viral infections of the respiratory tract we co-stimulated bronchial fibroblasts with CSE and Poly I:C and measured the production of pro-inflammatory and remodelling cytokines. In view of the results described above (section 3.4) it was decided that we should use a concentration range of Poly I:C from 0 – 0.1 μg/ml for these experiments. We had elected not to use a concentration of 0.5 μg/ml of Poly I:C as this concentration of Poly I:C significantly increases the expression of IL-6. Primary bronchial fibroblasts were cultured in 12 well tissue culture plates and were exposed to 0.01 μg/ml, 0.05 μg/ml and 0.1 μg/ml of Poly I:C with or without the presence of 2.5% CSE. Supernatants were harvested at 24 hours and VEGF and IL-6 expression was measured using ELISA. In view of the fact that Poly I:C alone did not demonstrate a trend for a concentration-dependent increase in spontaneous expression of TGF-β1 (in the concentrations tested) we did not examine TGF-β1 expression.

As before, Poly I:C alone in the concentration range 0 – 0.1 μg/ml did not significantly alter the secretion of VEGF over 24 hours. In the presence of 2.5% CSE, however, Poly I:C at a concentration of 0.1 μg/ml significantly increased VEGF production ($p = 0.0456$) and there was evidence of a concentration-dependent synergistic increase in the presence of 2.5% CSE (Anova $p = 0.4169$) (Figure 17).

While Poly I:C alone demonstrated a trend for a concentration dependent increase in IL-6 production by primary bronchial fibroblasts ($p = 0.0565$), the additional presence of 2.5% CSE did not demonstrate a clear trend of its ability to alter the effect of Poly I:C on the expression of IL-6 (Anova $p = 0.6156$) (Figure 18).

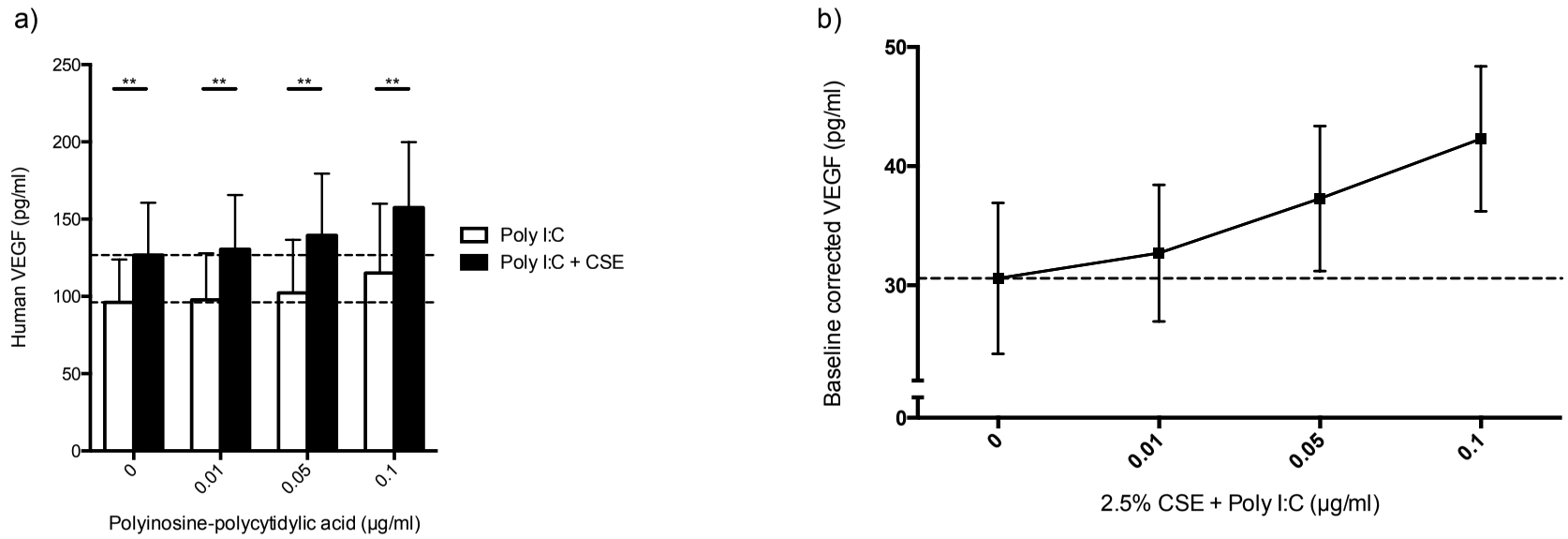

Figure 17: Co-stimulation of primary bronchial fibroblasts with polyinosinic:polycytidylic and cigarette smoke – VEGF

Primary bronchial fibroblasts were cultured in 12 well tissue culture plates as described in section 2.3.1.5 with a concentration series of Poly I:C (0, 0.01, 0.05 and 0.1 µg/ml) with or without 2.5% CSE. a) CSE synergistically increased Poly I:C-induced VEGF secretion (Poly I:C without vs with CSE, 0 µg/ml 96.12 pg/ml ± 27.69 vs 126.7 ± 33.91, p = 0.0085; 0.01 µg/ml 97.76 pg/ml ± 30.09 vs 130.5 ± 35.17, p = 0.0047; 0.05 µg/ml 102.2 pg/ml ± 34.44 vs 139.5 ± 39.93, p = 0.0036; 0.1 µg/ml 115.1 pg/ml ± 44.80 vs 157.4 ± 42.40, p = 0.0022). b) Concentration-dependent effect trend of Poly I:C on VEGF secretion in the presence of 2.5% CSE (Baseline 30.58 pg/ml ± 6.333; 0.1 µg/ml 32.70 pg/ml ± 5.739; 0.5 µg/ml 37.28 pg/ml ± 6.087; 1 µg/ml 42.29 pg/ml ± 6.081; Anova p = 0.4169). Paired t-test ** $p < 0.01$, Mean ± SEM, n = 5

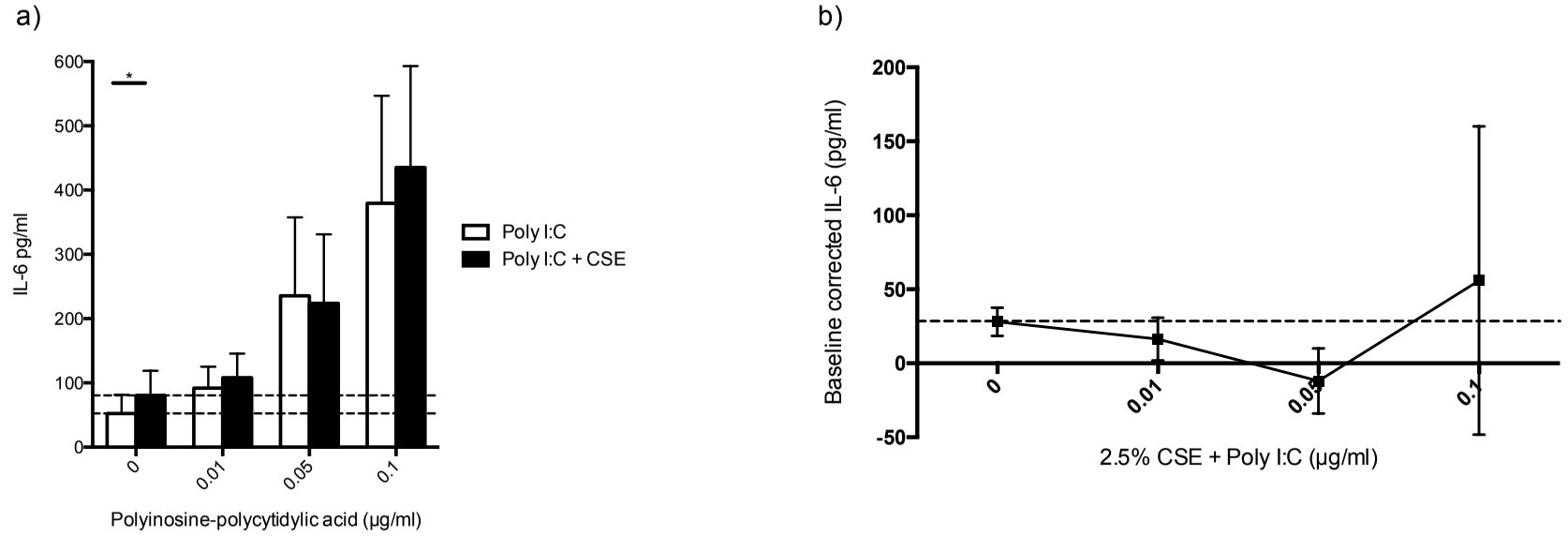

Figure 18: Co-stimulation of primary bronchial fibroblasts with polyinosinic:polycytidylic and cigarette smoke – IL-6

Primary bronchial fibroblasts were cultured in 12 well tissue culture plates as described in section 2.3.1.5 with a concentration series of Poly I:C (0, 0.01, 0.05 and 0.1 µg/ml) with or without 2.5% CSE. a) There was no clear effect of CSE on the expression of IL-6 when primary bronchial fibroblasts were stimulated with Poly I:C (Poly I:C without vs with CSE, 0 µg/ml 52.39 pg/ml ± 28.97 vs 80.45 ± 38.29, p = 0.0324; 0.01 µg/ml 91.76 pg/ml ± 33.54 vs 108.1 ± 37.46, p = 0.3107; 0.05 µg/ml 235.7 pg/ml ± 121.8 vs 223.7 ± 107.5, p = 0.6106; 0.1 µg/ml 379.4 pg/ml ± 167.3 vs 435.4 ± 157.7, p = 0.6142). b) As the concentration of Poly I:C increased, there was no clear trend identified of its ability to alter the effect of 2.5% CSE on the expression of IL-6 (Baseline 28.06 pg/ml ± 9.557; 0.1 µg/ml 16.35 pg/ml ± 14.50; 0.5 µg/ml -11.96 pg/ml ± 22.04; 1 µg/ml 55.92 pg/ml ± 104.1; Anova p = 0.6156). Paired t-test * $p < 0.05$, Mean ± SEM, n = 5

3.5 Effects of IL-17A on cigarette smoke extract induced expression of pro-inflammatory and remodelling cytokines

In view of our hypothesis that cigarette smoking causes structural cells to release cytokines which produce an environment that promotes the development of Th17 cells, we investigated whether interleukin-17A (IL-17A), a signature cytokine of Th17 cells is able to alter the expression of pro-inflammatory and remodelling cytokines induced by cigarette smoke extract in primary bronchial fibroblasts. A concentration of 10 ng/ml of IL-17A was shown to be effective at stimulating the release of IL-6 by synovial fibroblasts, while a concentration of 1 ng/ml was found to have no effect (Chabaud et al., 1998). Consequently, primary bronchial fibroblasts were cultured in 12 well tissue culture plates and exposed to 0.1 ng/ml, 1 ng/ml and 10 ng/ml of IL-17A with or without 2.5% CSE. Supernatants were harvested at 24 hour and VEGF and IL-6 expression was measured using ELISA.

IL-17A alone in the concentration range 0 – 10 ng/ml did not significantly alter spontaneous production of VEGF by cultured primary bronchial fibroblasts (Anova $p = 0.1336$). The additional presence of 2.5% CSE further augmented VEGF production but this effect was additive (Anova $p = 0.0578$) (Figure 19).

While IL-17A alone demonstrated a concentration dependent increase in IL-6 production by primary bronchial fibroblasts (Anova $p = 0.0011$), the additional presence of 2.5% CSE further augmented IL-6 production in a concentration related, synergistic fashion (Anova $p = 0.0023$) (Figure 20).

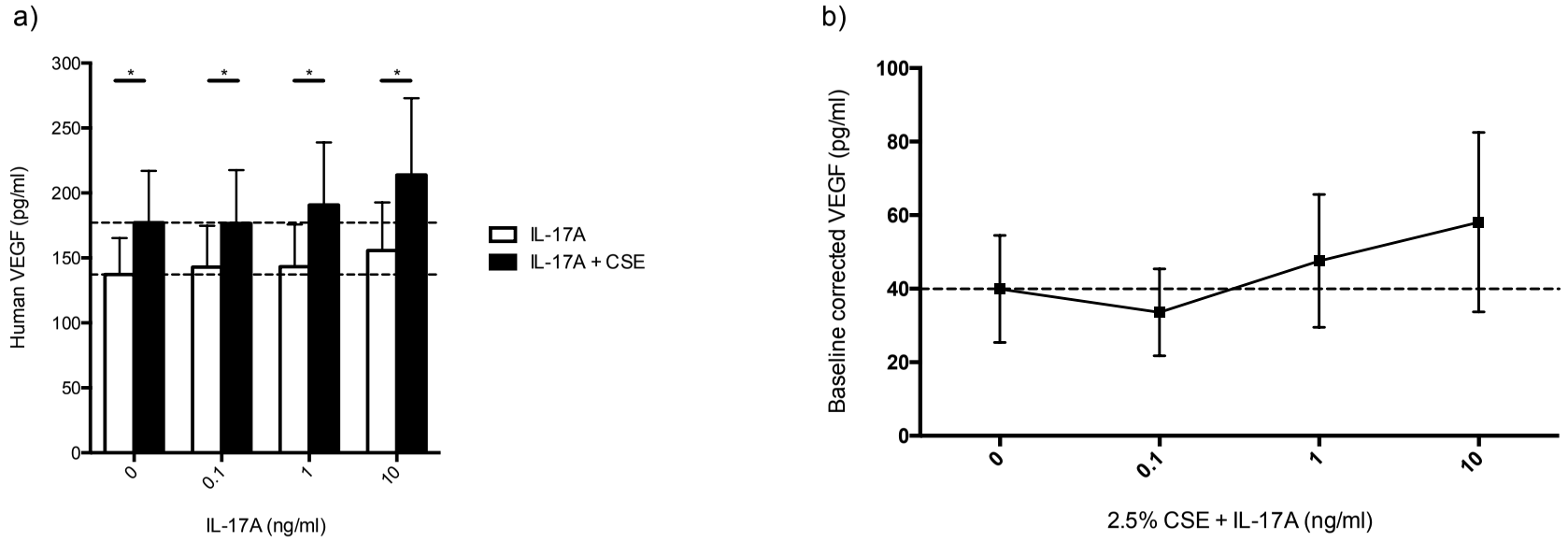

Figure 19: Co-stimulation of primary bronchial fibroblasts with IL-17A and cigarette smoke – VEGF

Primary bronchial fibroblasts were cultured in 12 well tissue culture plates as described in section 2.3.1.6 with a concentration series of IL-17A (0, 0.1, 1 and 10 ng/ml) with or without 2.5% CSE. a) CSE additively increased IL-17A-induced VEGF secretion (IL-17A without vs with CSE, 0 µg/ml 137.2 pg/ml ± 28.14 vs 177.1 ± 39.95, p = 0.0228; 0.1 ng/ml 142.9 pg/ml ± 31.83 vs 176.5 ± 41.08, p = 0.0194; 1 ng/ml 143.3 pg/ml ± 32.57 vs 190.8 ± 48.10, p = 0.0273; 10 ng/ml 155.7 pg/ml ± 37.08 vs 213.8 ± 59.06, p = 0.0412). b) The magnitude of effect of 2.5% CSE on the expression of VEGF was similar throughout the concentration range of IL-17A used (Baseline 39.94 pg/ml ± 14.57; 0.1 ng/ml 33.59 pg/ml ± 11.83; 1 ng/ml 47.57 pg/ml ± 18.08; 10 µg/ml 58.09 pg/ml ± 24.40, Anova p = 0.0578). Paired t-test * $p < 0.05$, Mean ± SEM, n = 8

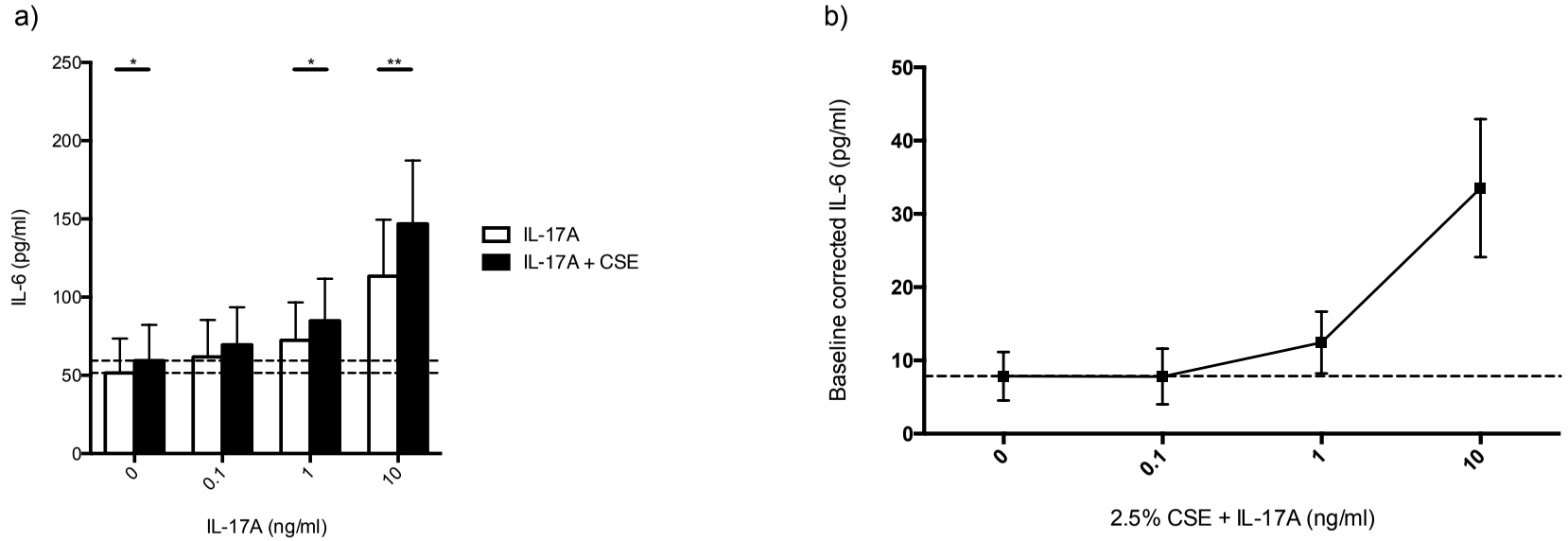

Figure 20: Co-stimulation of primary bronchial fibroblasts with IL-17A and cigarette smoke – IL-6

Primary bronchial fibroblasts were cultured in 12 well tissue culture plates as described in section 2.3.1.6 with a concentration series of IL-17A (0, 0.1, 1 and 10 ng/ml) with or without 2.5% CSE a) CSE synergistically increased IL-17A-induced IL-6 secretion (IL-17A without vs with CSE, 0 µg/ml 51.50 pg/ml ± 22.06 vs 59.35 ± 22.93, $p = 0.0493$; 0.1 ng/ml 61.76 pg/ml ± 23.68 vs 69.57 ± 24.00, $p = 0.0785$; 1 ng/ml 72.40 pg/ml ± 24.29 vs 84.85 ± 26.95, $p = 0.0214$; 10 ng/ml 113.4 pg/ml ± 36.16 vs 146.9 ± 40.50, $p = 0.0092$). b) Concentration-dependent effect of IL-17A on IL-6 secretion in the presence of 2.5% CSE (Baseline 7.854 pg/ml ± 3.308; 0.1 ng/ml 7.811 pg/ml ± 3.794; 1 ng/ml 12.45 pg/ml ± 4.219; 10 ng/ml 33.51 pg/ml ± 9.417, Anova $p = 0.0023$). Paired t-test * $p < 0.05$, ** $p < 0.01$, Mean ± SEM, $n = 8$

3.6 Signal transduction pathway of cigarette smoke extract induced expression of pro-inflammatory and remodelling cytokines

To investigate the signalling pathways through which CSE induces the expression of VEGF, IL-6 and TGF-β1 in primary bronchial fibroblasts, these cells were cultured with CSE and various intracellular inhibitors. We investigated the involvement of the Nuclear Factor Kappa-light-chain-enhancer of activated B cells (NF-κB) signalling pathway, Phosphatidylinositide 3-kinase (PI3K) signalling pathway and Mitogen-activated protein (MAP) Kinase signalling pathways in CSE induced expression of these cytokines.

To inhibit the NF-κB signalling pathway primary bronchial fibroblasts were cultured with 2.5% CSE and 2.5 μM BAY 11-7082 (Lee et al., 2007), as described in section 2.3.1.7 . BAY 11-7082 is an irreversible inhibitor of cytokine-induced IκB-α phosphorylation. Phosphorylation of IκB-α results in its dissociation from NF-κB. This allows NF-κB to migrate into the nucleus and initiate gene transcription. By inhibiting IκB-α phosphorylation BAY 11-7082 prevents IκB-α from dissociating from NF-κB and its subsequent proteasomal degradation, thereby inhibiting NF-κB signalling.

We also investigated the role of the 3 main MAP Kinase signalling modules in CSE induced expression of these cytokines. The role of the MAPK/Erk signalling pathway was investigated by culturing primary bronchial fibroblasts with 2.5% CSE and 5 μM PD 98059 (Cheung et al., 2005), and 2.5% CSE and 10 μM U 0126 (Monick et al., 2005), as described in section 2.3.1.7. Activation of MEK1/2 by phosphorylation leads to the phosphorylation and activation of Erk1/2. The activation of Erk1/2 leads to the initiation of gene transcription of the genes associated with the MAPK/Erk signalling pathway. PD 98059 is a highly selective inhibitor for MEK1 kinase activation which binds to the inactive form of MEK1 and prevents its activation by upstream activators such as c-Raf. U 0126 is a highly selective inhibitor of both MEK1 and MEK2, inhibiting both active and inactive MEK1/2.

The role of the MAPK/p38 MAPK pathway was investigated by culturing primary bronchial fibroblasts with 2.5% CSE and 5 μM SB 203580 (Takahashi et al., 2007) as described in section 2.3.1.7. SB 203580 inhibits the p38 MAPK catalytic activity by competitive inhibition of the binding of ATP to p38 MAPK. This prevents the subsequent phosphorylation and activation of transcription factors and post-transcriptional regulating factors.

The role of the MAPK/JNK pathway was investigated by culturing primary bronchial fibroblasts with 2.5% CSE and 2.5 μM SP 600125 (Ju et al., 2002) as described in section 2.3.1.7. SP 600125 is a selective competitive inhibitor of JNK1, 2 and 3. It inhibits the phosphorylation of JNK and thereby inhibits its inactivation.

Upstream from these pathways is phosphatidylinositol 3 (PI3) kinase. PI3 kinase is involved in the signalling pathway of a multitude of cellular receptors. We cultured primary bronchial fibroblasts with 2.5% CSE and 10 μM LY 294002 (Kamata et al., 2004), as described in section 2.3.1.7. LY 294002 is a selective and competitive inhibitor of PI3 kinase which inhibits PI3 kinase-dependent Akt phosphorylation and kinase activity.

Primary bronchial fibroblasts were cultured in 12 well tissue culture plates with 2.5% CSE and the intracellular inhibitors above as described in section 2.3.1.7. For baseline control expression, cells were cultured with and without 2.5% CSE. The intracellular inhibitors were reconstituted and stored in dimethyl sulphoxide (DMSO). In view of this vehicle controls, consisting of DMSO in medium diluted to the same dilution factor used for the various intracellular inhibitors (i.e. 1/500, 1/1000, 1/2000, 1/40000), were used for comparison. The cells were cultured for up to 72 hours and supernatants harvested at 24 hours for expression of VEGF and IL-6, and at 72 hours for expression of TGF-β1.

3.6.1 Vascular endothelial growth factor signal transduction

The baseline expression of VEGF was 97.04 pg/ml ± 16.98 in the absence of 2.5% CSE and 152.2 pg/ml ± 32.25 in the presence of 2.5% CSE. Both SB 203580 and U 0126 were able completely to inhibit the expression of VEGF induced by 2.5% CSE (98.05 pg/ml ± 18.74, p = 0.0165; 86.70 pg/ml ± 13.88, p = 0.0249 respectively), while PD 98059 was able to reduce the expression of VEGF induced by 2.5% CSE (124.7 pg/ml ± 22.79, p = 0.0478). SP 600125 and LY 294002 had no effect on the expression of VEGF induced by 2.5% CSE (145.2 pg/ml ± 30.28, p = 0.2677; 126.7 pg/ml ± 29.18, p = 0.0714 respectively). BAY 11-7082 however significantly increased the expression of VEGF when primary bronchial fibroblasts were stimulated with 2.5% CSE (203.4 pg/ml ± 43.87, p = 0.0168). Vehicle control did not significantly affect baseline expression of VEGF (DMSO 1/500 dilution 94.44 pg/ml ± 14.71, p = 0.6739) (Figure 21).

3.6.2 Interleukin-6 signal transduction

The baseline expression of IL-6 was 62.92 pg/ml ± 32.10 in the absence of 2.5% CSE and 99.37 pg/ml ± 40.26 in the presence of 2.5% CSE. Both SB 203580 and U 0126 were able completely to inhibit the expression of IL-6 induced by 2.5% CSE (36.65 pg/ml ± 18.48, p = 0.0311; 26.70 pg/ml ± 12.05, p = 0.0389 respectively). There was a trend for the inhibition of expression of IL-6 induced by 2.5% with LY 294002 (55.78 pg/ml ± 20.91, p = 0.0694). PD 98059 and BAY 11-7082 had no effect on the expression of IL-6 induced by 2.5% CSE (93.68 pg/ml ± 50.80, p = 0.7574; 78.58 pg/ml ± 25.17, p = 0.4295 respectively). SP 600125 however significantly increased the expression of IL-6 when primary bronchial fibroblasts were stimulated with 2.5% CSE (140.1 pg/ml ± 51.76, p = 0.0159). Vehicle control did not significantly affect baseline expression of IL-6 (DMSO 1/500 dilution 98.05 pg/ml ± 45.69, p = 0.0996) (Figure 22).

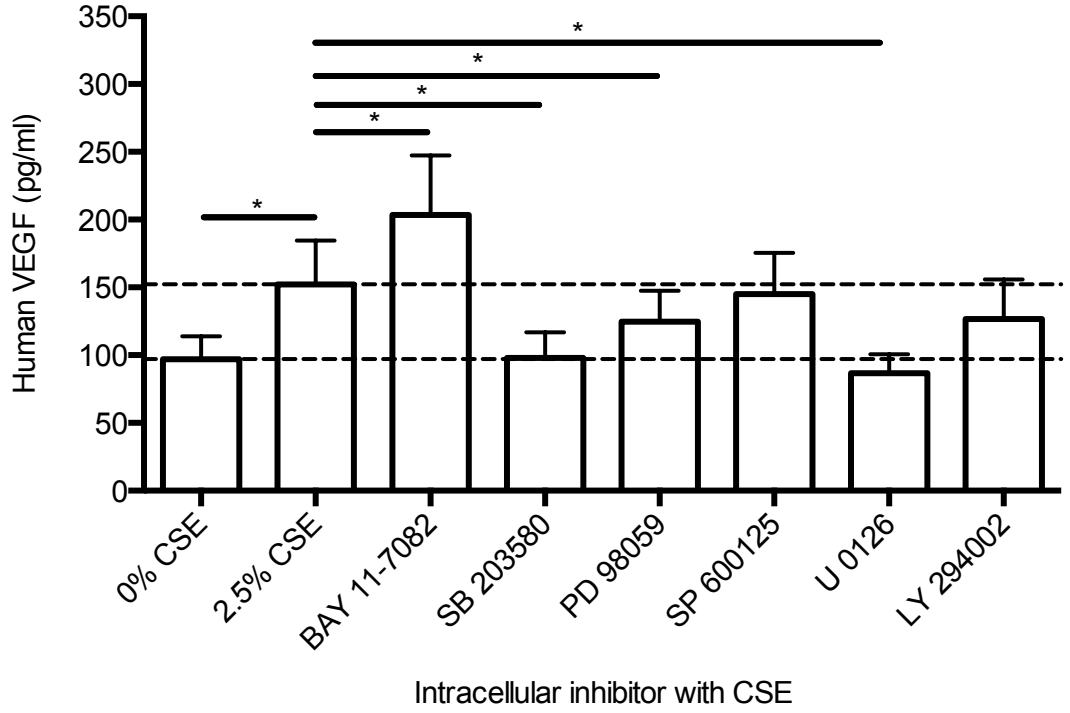

Figure 21: Primary bronchial fibroblast signal transduction – Vascular endothelial growth factor

Primary bronchial fibroblasts were cultured in 12 well tissue culture plates as described in section 2.3.1.7 with 2.5% CSE and various intracellular inhibitors. 2.5% CSE significantly increased the expression of VEGF (0% CSE 97.04 pg/ml ± 16.98 vs 2.5% CSE 152.2 pg/ml ± 32.25, p = 0.0333). This was completely inhibited by SB 203580 and U 0126 (98.05 pg/ml ± 18.74, p = 0.0165; 86.70 pg/ml ± 13.88, p = 0.0249 respectively) and significantly reduced with PD 98059 (124.7 pg/ml ± 22.79, p = 0.0478). SP 600125 and LY 294002 had no effect on the expression of VEGF induced by 2.5% CSE (145.2 pg/ml ± 30.28, p = 0.2677; 126.7 pg/ml ± 29.18, p = 0.0714 respectively). BAY 11-7082 however significantly increased the expression of VEGF when primary bronchial fibroblasts were stimulated with 2.5% CSE (203.4 pg/ml ± 43.87, p = 0.0168). Vehicle control had no significant affect on the baseline expression of VEGF (DMSO 1/500 dilution 94.44 pg/ml ± 14.71, p = 0.6739). Paired t-test * $p < 0.05$, Mean ± SEM, n = 5

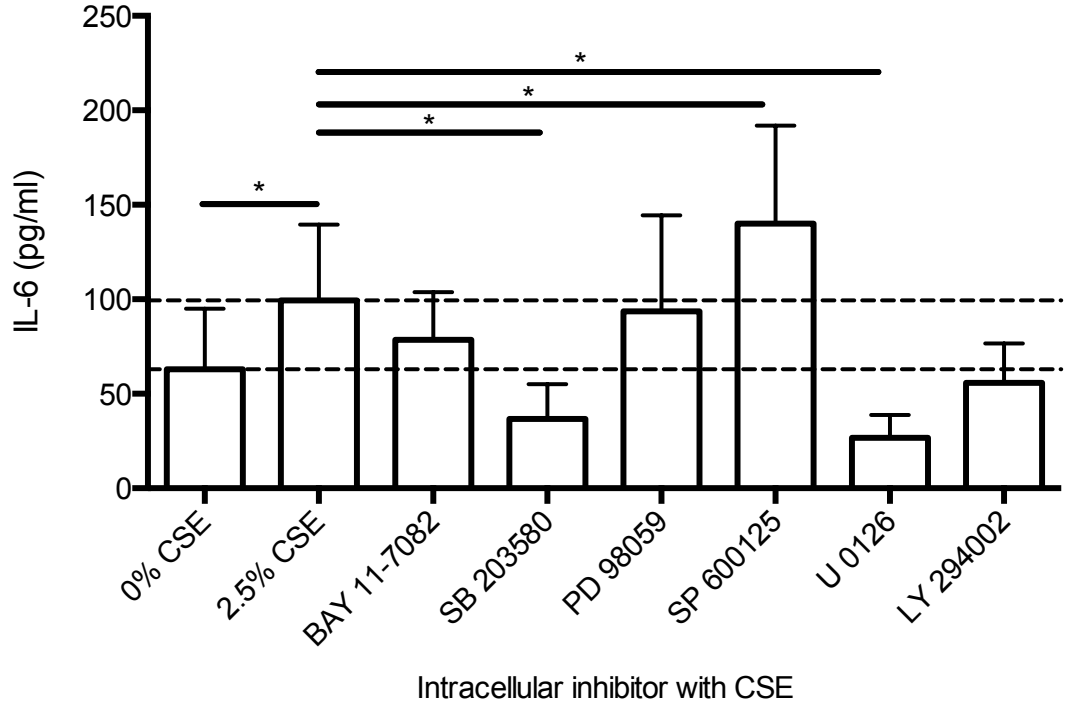

Figure 22: Primary bronchial fibroblast signal transduction – IL-6

Primary bronchial fibroblasts were cultured in 12 well tissue culture plates as described in section 2.3.1.7 with 2.5% CSE and various intracellular inhibitors. 2.5% CSE significantly increased the expression of IL-6 (0% CSE 62.92 pg/ml ± 32.10 vs 2.5% CSE 99.37 pg/ml ± 40.26, $p = 0.0303$). This was completely inhibited by SB 203580 and U 0126 (36.65 pg/ml ± 18.48, $p = 0.0311$; 26.70 pg/ml ± 12.05, $p = 0.0389$ respectively) and a trend of inhibition was seen with LY 294002 (55.78 pg/ml ± 20.91, $p = 0.0694$). PD 98059 and BAY 11-7082 had no effect on the expression of IL-6 induced by 2.5% CSE (93.68 pg/ml ± 50.80, $p = 0.7574$; 78.58 pg/ml ± 25.17, $p = 0.4295$ respectively). SP 600125 however significantly increased the expression of IL-6 when primary bronchial fibroblasts were stimulated with 2.5% CSE. Vehicle control had no significant affect on the baseline expression of IL-6 (DMSO 1/500 dilution 98.05 pg/ml ± 45.69, $p = 0.0996$). Paired t-test * $p < 0.05$, Mean ± SEM, $n = 8$

3.6.3 Transforming growth factor beta 1 signal transduction

The baseline expression of TGF-β1 was 396.7 pg/ml ± 135.6 in the absence of 2.5% CSE and 431.0 pg/ml ± 144.1 in the presence of 2.5% CSE. Both SP 600125 and U 0126 were able completely to inhibit the expression of TGF-β1 induced by 2.5% CSE (349.8 pg/ml ± 117.5, p = 0.0244; 284.1 pg/ml ± 84.36, p = 0.0487 respectively). SB 203580, PD 98059, BAY 11-7082 and LY 294002 had no significant effect on the expression of TGF-β1 induced by 2.5% CSE (407.9 pg/ml ± 145.5, p = 0.2807; 404.7 pg/ml ± 147.0, p = 0.0579; 421.1 pg/ml ± 150.7, p = 0.3618; 461.1 pg/ml ± 156.2, p = 0.2158 respectively). Vehicle control did not significantly affect baseline expression of TGF-β1 (DMSO 1/500 dilution 420.0 pg/ml ± 144.6, p = 0.1374) (Figure 23).

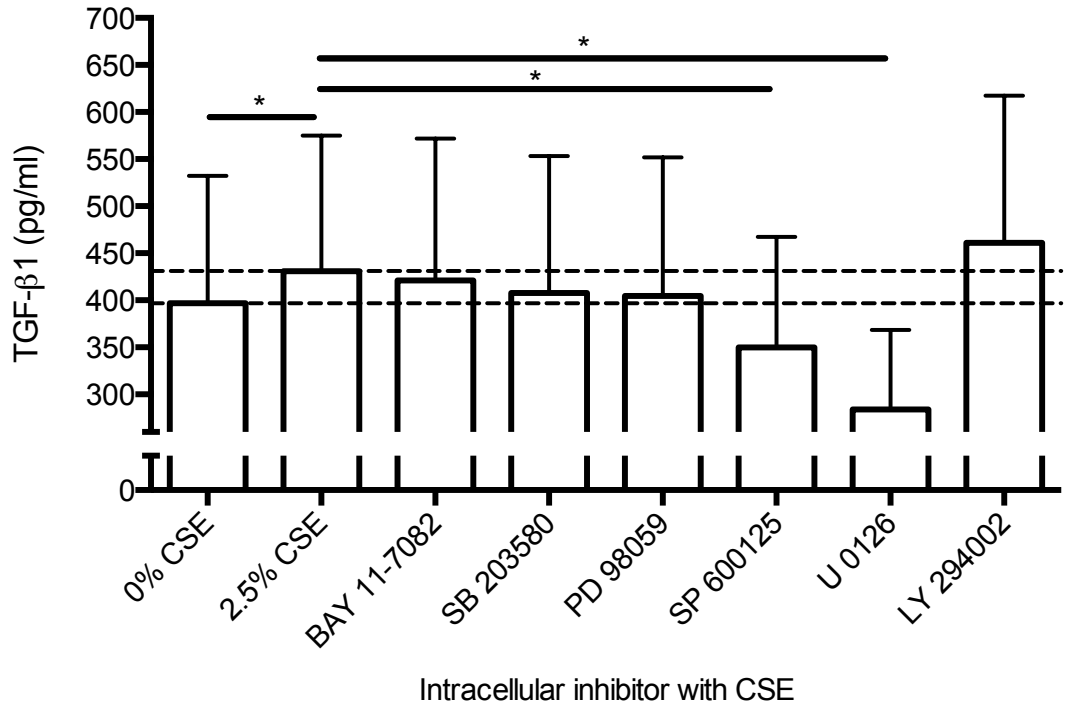

Figure 23: Primary bronchial fibroblast signal transduction – TGF-β1

Primary bronchial fibroblasts were cultured in 12 well tissue culture plates as described in section 2.3.1.7 with 2.5% CSE and various intracellular inhibitors. 2.5% CSE significantly increased the expression of TGF-β1 (0% CSE 396.7 pg/ml ± 135.6 vs 2.5% CSE 431.0 pg/ml ± 144.1, p = 0.0335). This was completely inhibited by SP 600125 and U 0126 (349.8 pg/ml ± 117.5, p = 0.0244; 284.1 pg/ml ± 84.36, p = 0.0487 respectively). SB 203580, PD 98059, BAY 11-7082 and LY 294002 had no significant effect on the expression of TGF-β1 induced by 2.5% CSE (407.9 pg/ml ± 145.5, p = 0.2807; 404.7 pg/ml ± 147.0, p = 0.0579; 421.1 pg/ml ± 150.7, p = 0.3618; 461.1 pg/ml ± 156.2, p = 0.2158 respectively). Vehicle control did not significantly affect baseline expression of TGF-β1 (DMSO 1/500 dilution 420.0 pg/ml ± 144.6, p = 0.1374). Paired t-test * $p < 0.05$, Mean ± SEM, n = 8

3.7 Discussion and summary

Because of the potential toxic effects of CSE we considered it important to monitor cellular viability during the experiments. Trypan blue exclusion studies on adherent cells partially fulfilled this function but we had the impression that affected cells were detaching from the culture plate and so we also monitored cellular confluence. Attempts to back up these observations by documenting annexin-V/PI staining by flow cytometry were similarly complicated by the tendency of the cells to clump, making it difficult to define a clear cellular population for analysis based on size/scatter characteristics. At the conclusion we determined with both methods that concentrations of 10% CSE or above are likely to be significantly toxic to the fibroblasts. The data are consistent with previous and recently published literature using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) incorporation to assess cellular viability (Yang et al., 2013, Baglole et al., 2006, Park et al., 2010).

Primary bronchial fibroblasts spontaneously secreted VEGF, IL-6 and TGF-β1 into the culture media ex vivo and this was significantly enhanced in a concentration-dependent fashion by CSE at concentrations which did not compromise cellular viability. Interestingly, bFGF was not spontaneously secreted by the fibroblasts but was induced in the presence of high concentrations of CSE, which would have been expected to compromise cellular viability, suggesting that its secretion is associated with cell damage and possibly the process of apoptosis and/or necrosis. We believe this to be the first such demonstration.

During the course of this body of work, Volpi et al. in 2011 published their findings that CSE is able to induce the expression of VEGF in normal human lung fibroblasts and airway smooth muscle cells, and that this expression is mediated via the p38 MAPK signalling pathway (Volpi et al., 2011). We have here however demonstrated that inhibition of either the p38 MAPK or the ERK signalling pathways causes complete suppression of CSE induced VEGF expression in primary bronchial fibroblasts. The involvement of both p38 MAPK and ERK signalling pathways in CSE mediated cytokine release by normal human lung fibroblasts, has also been

shown in the case of CSE induced IL-8 expression (Moretto et al., 2009). This reaffirms the present findings that not only is p38 MAPK signalling required, but the ERK pathway also plays a critical role for CSE to induce VEGF expression. A further intriguing finding was that inhibition of the NF-κB signalling pathway results in increased expression of VEGF when primary bronchial fibroblasts are stimulated with CSE. This finding would suggest that the NF-κB signalling pathway plays an inhibitory role in CSE induced VEGF expression. The relevance of this finding is unclear at present and further investigation is warranted.

CSE-induced IL-6 production has previously been demonstrated in human fibroblast-like synoviocytes (Shizu et al., 2008) and in response to the cigarette-smoke carcinogen benzo[a]pyrene diol epoxide in human foetal lung fibroblast (HFL-1) cell lines (Chen et al., 2012). We believe that this is the first demonstration of CSE inducing IL-6 in adult primary bronchial fibroblasts. As with CSE induced VEGF expression, both p38 MAPK and ERK signalling pathways were required for CSE induced expression of IL-6. Further, the data demonstrate that the PI3 kinase signalling pathway may also play a role in CSE induced expression of IL-6. Benzo[a]pyrene diol epoxide induction of IL-6 in HFL-1 cells was shown to be dependent on both NF-κB and ERK signalling pathways (Chen et al., 2012). In contrast the present data suggest that the NF-κB signalling pathway is not involved in the induction of IL-6 by CSE in primary bronchial fibroblasts. This difference could be due either to benzo[a]pyrene diol epoxide being a single constituent of over 4000 chemicals in CSE or to differences between foetal and adult lung fibroblasts, or both. Another intriguing finding is that inhibition of the JNK signalling pathway results in increased expression of IL-6 when primary bronchial fibroblasts are stimulated with CSE. This finding would suggest that the JNK signalling pathway plays an inhibitory role in CSE induced IL-6 expression. Again the relevance of this finding is unclear and further investigation is warranted.

CSE induction of TGF-β1 has previously been demonstrated in human foetal lung fibroblasts (HFL-1) (Wang et al., 2003, Ge et al., 2004), but this is the first demonstration of CSE inducing TGF-β1 in adult primary bronchial fibroblasts. The "basal" expression of TGF-β1 in our experiments may partially be accounted for by

the fact that 1% foetal bovine serum, which contains TGF-β1, was included in the culture medium. Wang et al. showed that the expression of TGF-β1 in response to CSE in vitro was related to cell density, and that there was no difference in the expression of total TGF-β1 when HFL-1 were stimulated by CSE, but an increase in "active" TGF-β1 (Wang et al., 2003). We found a modest increase in total TGF-β1 following exposure to CSE, and in hindsight a larger difference between baseline and stimulation with CSE could have been observed had we measured only "active" TGF-β1 rather than total TGF-β1.

In contrast to VEGF and IL-6 induction by CSE, the induction of TGF-β1 by CSE in primary bronchial fibroblasts was dependent on both the ERK and JNK signalling pathways with the possible involvement of the p38 MAPK pathway. Considering the difference in the time course of expression of both VEGF and IL-6 compared to TGF-β1, one could postulate that CSE initially activates both the ERK and p38 MAPK pathways resulting in the release of VEGF and IL-6, then over time either due to the reduction in the oxidative stress potential or due to the varying activity of the components of CSE, this results in the late activation of the JNK signalling pathway resulting in TGF-β1 expression.

Cigarette smoking has been shown to cause resistance to treatment to both inhaled (Chalmers et al., 2002, Tomlinson et al., 2005) and oral (Chaudhuri et al., 2003) corticosteroids in asthma. In view of this we investigated whether the pro-inflammatory and pro-remodelling cytokines induced by CSE in primary bronchial fibroblasts could be inhibited by corticosteroids. We demonstrated that dexamethasone was able completely to suppress the release of VEGF, IL-6 and TGF-β1 induced by CSE. It is thus unlikely that these phenomena form a component of the clinical resistance to corticosteroid therapy described in smoking asthmatics, at least with regard to the effects of CSE on fibroblasts.

Smoking asthmatic patients have poor symptom control and a higher rate of asthma exacerbations compared to non-smoking asthmatics. Furthermore smoking as well as viral respiratory tract infections are recognised trigger factors for asthma exacerbation (Dougherty and Fahy, 2009, Talreja et al., 2012, Polosa and Thomson, 2013).

Cigarette smoke has been shown to be able to attenuate the immune response triggered by viral infections in both structural and immune cells. In normal bronchial epithelial cells, it is able to inhibit the induction of CXCL10, CCL5 and IFN-β, while increasing viral replication (Eddleston et al., 2011). In human lung fibroblasts, cigarette smoke is able to inhibit both the release of type I interferons in response to Poly I:C stimulation but also the response of fibroblasts to stimulation with IFN-β (Bauer et al., 2008). Furthermore cigarette smoke also suppresses the release of type I interferons when peripheral blood mononuclear cells are stimulated with Poly I:C. The function of NK cells in response to Poly I:C is also attenuated by cigarette smoke (Mian et al., 2008, Mian et al., 2009b, Mian et al., 2009a).

In the context of bacterial infections, Pace et al showed that CSE augments the release of IL-8, while inhibiting the release of IP-10, when bronchial epithelial cells are stimulated with lipopolysaccharide (LPS) (Pace et al., 2008). Prior to this Laan et al showed that CSE inhibited the release of IL-8 and GM-CSF when bronchial epithelial cells are stimulated with LPS (Laan et al., 2004). The conflicting data from these two groups might be attributable to differences in the CSE preparation and that higher concentration of CSE caused inhibition while lower concentrations of CSE were stimulatory. CSE conditioned dendritic cells have been shown preferentially to inhibit the Th1 immune response. CSE inhibited LPS-induced release of IL-12p70, with an enhancement of IL-10 release and no effect on IL-6 release. Furthermore these CSE conditioned dendritic cells in mixed-lymphocyte reactions caused a corresponding reduction in IFN-γ release, with an enhancement of IL-4 and IL-10 release, and no effect on IL-2 release (Vassallo et al., 2005).

Looking for possible interactions of cigarette smoking with viral and bacterial infections of the respiratory tract, primary bronchial fibroblasts were first stimulated with LTA, Poly I:C and LPS to see whether any of these TLR agonists are able to induce the expression of VEGF, IL-6 and TGF-β1. We found that LTA and Poly I:C were able to induce IL-6 and VEGF expression, but not TGF-β1. Despite stimulating the primary bronchial fibroblasts with up to 10 μg/ml of LPS it was not possible to show any induction of IL-6 expression (data not shown). Both LTA and Poly I:C were able to augment the expression of VEGF induced by CSE in a synergistic manner and

the expression of IL-6 induced by CSE in an additive manner. These data suggest that CSE may be able to enhance the airways inflammatory response seen following viral or bacterial infections, despite inhibiting both the innate response from structural cells and the cell mediated Th1 immune response.

The data further clearly show that primary bronchial fibroblasts release both IL-6 and TGF-β1 in response to CSE, and that surrogates of bacterial and viral infection further enhance this. The resulting cytokine release by the primary bronchial fibroblasts may be postulated to create an environment in the bronchial submucosa that promotes the development of Th17 cells (Zuniga et al., 2013, Muranski and Restifo, 2013). We investigated this further by obtaining endobronchial biopsies from smoking and non-smoking asthmatics as well as from healthy controls, the results of which will be presented in Chapter 5.

IL-17A is one of the signature cytokines of a Th17 cell. In rheumatoid arthritis the interaction between human fibroblast-like synoviocytes and Th17 cells has been shown to increase the expression of IL-17A, resulting in the generation of a positive feedback loop stimulating the fibroblast-like synoviocytes to release the pro-inflammatory cytokines IL-6 and IL-8. Inhibition of the cyclooxygenase/prostaglandin E2 pathway or neutralisation of IL-17A or TNF-α have been shown significantly to inhibit the release of IL-6 and IL-8 (van Hamburg et al., 2011, Paulissen et al., 2013). Human fibroblast-like synoviocytes have also been shown to release VEGF in response to stimulation with IL-17A (Ryu et al., 2006, Honorati et al., 2006). Human lung fibroblasts from healthy and asthmatic patients have been shown to release IL-6, IL-8, IL-11 and CXCL1 in response to stimulation with IL-17A (Molet et al., 2001). We have demonstrated that IL-17A is able to augment the expression of both IL-6 and VEGF induced by CSE in a synergistic manner. Therefore CSE may play an important role in the perpetuation of Th17 induced inflammation.

In summary we have demonstrated that CSE is able to promote the release of a cytokine milieu by primary bronchial fibroblasts that supports the development of Th17 type inflammation. The perpetuation of this cytokine milieu is also supported by

the synergistic effects of CSE on the response of primary bronchial fibroblasts to LTA, Poly I:C and IL-17A.

Chapter 4: Bronchial epithelial cells *in vitro* experiments

4 Bronchial epithelial cells *in vitro* experiments

4.1 Introduction

Bronchial epithelial cells function not only as a physical barrier to the environment but also play a role in immune surveillance for potential pathogens (Rate et al., 2012). They are the initial contact point for pathogens and allergens in the respiratory tract and are able to respond to them through pattern recognition receptors (Lebedev and Poniakina, 2006, Lambrecht and Hammad, 2013).

4.1.1 Bronchial epithelial cells in asthma

In asthma there is an increase in epithelial layer fragility resulting in epithelial desquamation (Gleich et al., 1987). There is also goblet cell hyperplasia and increased mucus production (Dunnill et al., 1969). Furthermore the barrier function of bronchial epithelial cells in asthma is reduced with patchy disruptions of the tight junctions (Xiao et al., 2011). Epithelial cells also contribute to airways remodelling by expressing VEGF, periostin and TGF-β, which are stimuli for angiogenesis and collagen I deposition (Sidhu et al., 2010, Lopez-Guisa et al., 2012).

Bronchial epithelial cells also release pro-inflammatory cytokines in response to exposure to allergens, irrespective of whether the allergen exhibits protease activity (Tomee et al., 1998, King et al., 1998, Kauffman et al., 2006, Roschmann et al., 2009, Osterlund et al., 2009, Osterlund et al., 2011). In asthmatic airways there is increased expression of thymic stromal lymphopoietin (TSLP) in bronchial epithelial cells (Ying et al., 2005), and on exposure to allergen they are the initial source of TSLP produced in an allergic response (Corrigan et al., 2009). Furthermore they respond to the Th2 signature cytokines IL-4 and IL-13 to release GM-CSF, IL-8, CCL11/Eotaxin and CCL17/TARC (Matsukura et al., 2001, Terada et al., 2001, Lordan et al., 2002).

4.1.2 Effects of cigarette smoke exposure on bronchial epithelial cells

Bronchial epithelial cells exposed to cigarette smoke are stimulated to release pro-inflammatory cytokines, which leads to an influx of neutrophils into the vicinity (Wyatt et al., 1999, Kode et al., 2006). Cigarette smoke also disrupts the epithelial

tight junctions, facilitating the trans-epithelial passage of viruses, bacteria and allergen (Rusznak et al., 1999, Petecchia et al., 2009, Schamberger et al., 2013). Epithelial cells from asthmatic subjects have been shown to be more sensitive compared to healthy controls to this disruption (Xiao et al., 2011). Furthermore cigarette smoke also generates reactive oxidative species in the epithelial cells causing DNA damage and subsequent release of damage-associated molecular patterns (DAMPs) and cellular death (Liu et al., 2009). Cigarette smoke also reduces the ability of bronchial epithelial cells to "respond" to viral infections (Castro et al., 2008, Hudy et al., 2010, Eddleston et al., 2011, Proud et al., 2012).

4.1.3 Summary

Bronchial epithelial cells play a key role as the "first" responders to pathogenic, noxious and allergenic stimuli. They also aid in the perpetuation of an inflammatory response by the release of pro-inflammatory cytokines and chemokines.

The objective of the experiments described in this chapter was to investigate the role that bronchial epithelial cells may play in the induction and maintenance of the hypothesized IL-17A mediated neutrophilic inflammation induced by cigarette smoking in asthma and to investigate the interaction between IL-17A, CSE and allergen in bronchial epithelial cells. A further objective was to investigate the role that bronchial epithelial cells may play in the hypothesized increased angiogenesis induced by cigarette smoking in asthma. These objectives were addressed by performing in vivo experiments on human tracheal epithelial cells (HTEpC) with the aim of addressing the following hypotheses:

Neutrophilia hypothesis

1. Cigarette smoke extract stimulates human tracheal epithelial cells to produce IL-17A and IL-17F.
2. Cigarette smoke extract is able to modulate the spontaneous release of TSLP by human tracheal epithelial cells.
3. Cigarette smoke extract is able to enhance the pro-inflammatory cytokines produced by IL-17A stimulation of human tracheal epithelial cells.
4. Reactive oxygen species may be involved in the mechanism underlying the enhancement of pro-inflammatory cytokines produced by IL-17A stimulation of human tracheal epithelial cells.
5. Aeroallergens are able to modulate the synergistic/additive enhancement seen with co-stimulation with IL-17A and cigarette smoke extract of bronchial epithelial cells.
6. Cigarette smoke extract is able to attenuate the production of TSLP produced by TLR3 stimulation of bronchial epithelial cells.

Angiogenesis hypothesis

1. Cigarette smoke extract stimulates bronchial epithelial cells to produce the angiogenic growth factor vascular endothelial growth factor (VEGF).
2. Cigarette smoke extract is able to enhance VEGF secretion induced by IL-17A stimulation of bronchial epithelial cells.
3. Cigarette smoke induction of VEGF by bronchial epithelial cells involves discrete signalling pathways.

4.2 Effect on cigarette smoke extract on bronchial epithelial cells

Human Tracheal Epithelial Cells (HTEpC) were purchased from PromoCell (Cat No C-12644, PromoCell GmbH, Heidelberg, Germany). Experiments were conducted on cells from Passage 4 and 5, at 100% confluence, in 12 well tissue culture plates as described in section 2.4.1 above.

4.2.1 Epithelial cell viability

HTEpC were cultured in 12 well culture plates and exposed to a concentration series of CSE as described in section 2.4.1.1. The supernatants were removed and cellular viability assessed using trypan blue staining of the adherent cells. The percentage confluence was also estimated using an inverted light microscope at 10x magnification.

As in Chapter 3, section 3.2.1, adherent cells remained viable as assessed by trypan blue exclusion and lack of confluence was therefore used as an indicator of possible cellular damage. Cellular viability was maintained in HTEpC cultured for up to 72 hours and exposed to up to 2.5% CSE (Figure 24). Cellular viability was noted to be reduced in concentrations of CSE in excess of 2.5% (data not shown).

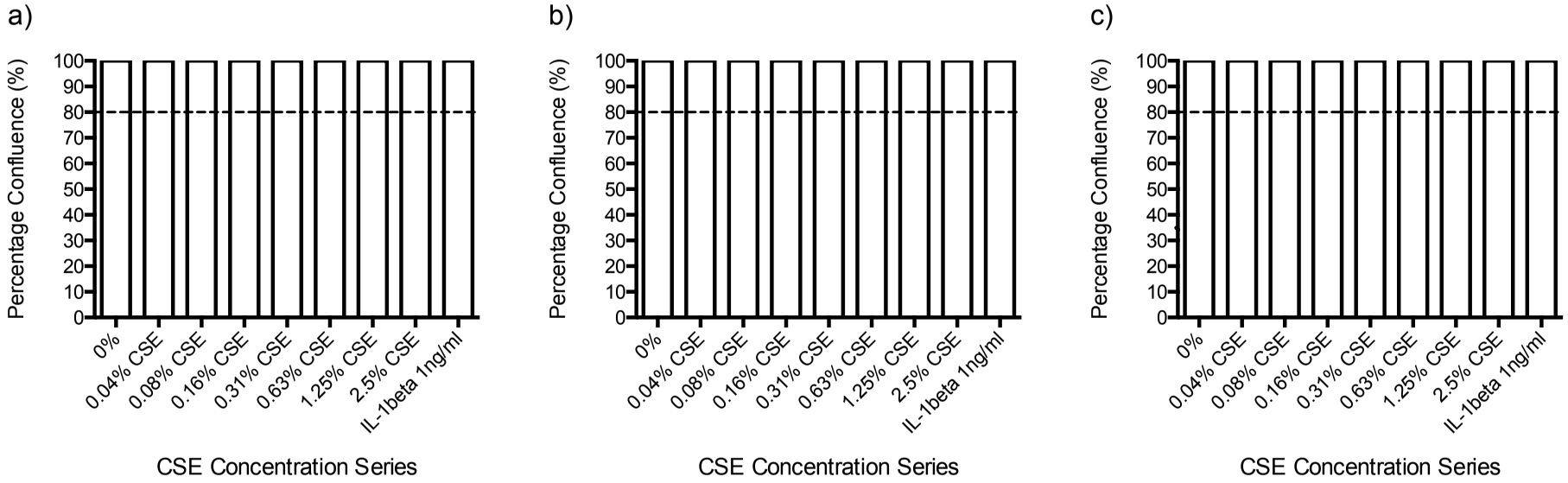

Figure 24: Viability of human tracheal epithelial cells (HTEpC) in the presence of CSE assessed using trypan blue

Human tracheal epithelial cells (HTEpC) were cultured in 12 well tissue culture plates and exposed to a concentration series of CSE as described in section 2.4.1.1. Viability and percentage confluence were measured using trypan blue staining at a) 24 hours, b) 48 hours and c) 72 hours. Percentage confluence and viability was maintained at 100% at all time points with concentrations below 2.5% CSE. There was no significant reduction in cellular viability and percentage confluence at all concentrations of CSE. n = 8.

4.2.2 Epithelial cell interleukin-6 expression

HTEpC were cultured in 12 well tissue culture plates and exposed to a concentration series of CSE, as described in section 2.4.1.1. Interleukin-6 (IL-6) release by HTEpC was measured using ELISA (as described in section 2.5) after 24 hours, 48 hours and 72 hours of culture. This experiment was done to optimise the culture conditions in view of the fact that it has been well described that human bronchial epithelial cells release IL-6 and IL-8 in response to CSE (Wyatt et al., 1999, Kode et al., 2006).

CSE induced IL-6 release in a concentration-dependent fashion after 24 and 72 hours of culture (Anova $p = 0.0026$ and $p = 0.0014$ respectively). CSE up to a concentration of 0.31% further increased spontaneous IL-6 release at all time points. No effect on spontaneous release of IL-6 was seen at 0.63% CSE, while concentrations above 0.63% reduced spontaneous release of IL-6. IL-1β stimulation was used as a positive control (Cromwell et al., 1992). At 24 hours the maximum increase of IL-6 was 1.430 ± 0.2517 fold above baseline, while that at 48 hours 1.171 ± 0.0404 fold above baseline and at 72 hours 1.481 ± 0.1125 fold above baseline (Mean ± SD) (Figure 25 and Table 2).

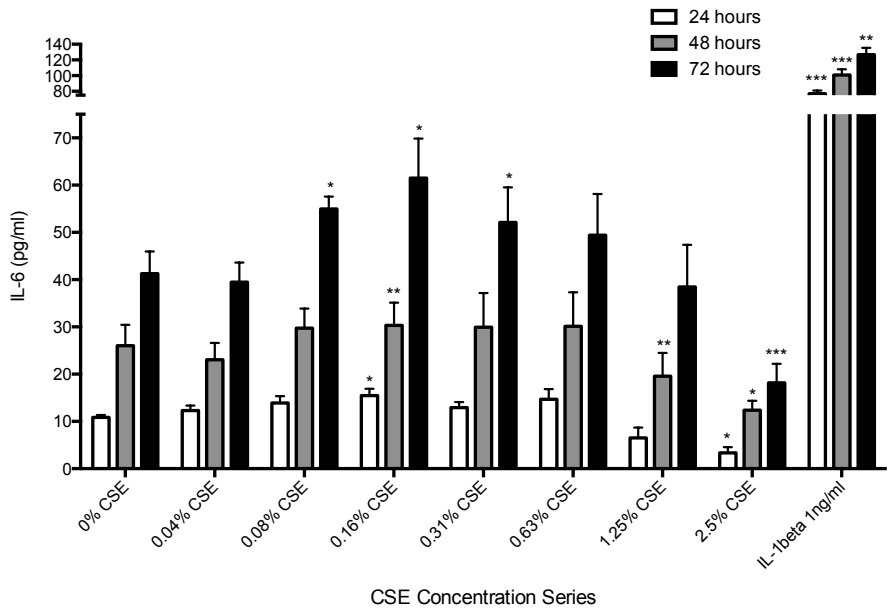

Figure 25: Influence of CSE on spontaneous IL-6 production by HTEpC

HTEpC were cultured in 12 well tissue culture plates as described in section 2.4.1.1 and exposed to a concentration series of CSE. When stimulated with CSE, IL-6 was released in a concentration dependent manner at 24 and 72 hours of culture (Anova p = 0.0026 and p = 0.0014 respectively). Paired t-test * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Mean ± SEM, n = 4

Time Course (hours)	Baseline	Concentration of Cigarette Smoke Extract							IL-1β 1 ng/ml	Anova p value
		0.04%	0.08%	0.16%	0.31%	0.63%	1.25%	2.5%		
24	10.86 ± 0.5133	12.29 ± 1.048	13.90 ± 1.464	15.48 ± 1.452 †	12.93 ± 1.163	14.67 ± 2.160	6.496 ± 2.214	3.363 ± 1.202 †	77.04 ± 3.918 ‡	0.0026
48	26.03 ± 4.397	23.05 ± 3.569	29.76 ± 4.145	30.31 ± 4.810 §	29.92 ± 7.234	30.12 ± 7.190	19.57 ± 4.949 §	12.37 ± 2.000 †	100.7 ± 7.286 ‡	0.0624
72	41.29 ± 4.675	39.49 ± 4.119	54.96 ± 2.627 †	61.50 ± 8.375 †	52.09 ± 7.427 †	49.42 ± 8.694	38.45 ± 8.939	18.18 ± 4.016 ‡	126.7 ± 8.826 §	0.0014

IL-6 pg/ml Mean ± SEM, n = 4; Paired t-test † $p < 0.05$, § $p < 0.01$, ‡ $p < 0.001$ vs baseline

Anova comparing concentrations from baseline to 0.31% CSE

Table 6: Effect of CSE on the time course of spontaneous IL-6 production by HTEpC

The data obtained from the experiment conducted in section 4.2.2 were used to optimise the CSE exposure culture conditions for the following experiments. In view of this HTEpCs were exposed to up to 1.25% CSE and for up to 72 hours of culture, as described in section 2.4.1.1. Following 24, 48 and 72 hours of culture the supernatants were harvested and stored at -80^{o}C. VEGF, IL-17A, IL-17F and TSLP were measured using ELISA as described in section 2.5.

4.2.3 Epithelial cell vascular endothelial growth factor expression

HTEpC were cultured in 12 well tissue culture plates and exposed to a concentration series of CSE as described in section 2.4.1.1. Vascular endothelial growth factor (VEGF) release by the cells was measured using ELISA (as described in section 2.5) after 24 hours, 48 hours and 72 hours of culture. CSE induced VEGF release in a concentration-dependent fashion after 24 hours, 48 hours and 72 hours of culture (Anova $p < 0.0001$, $p < 0.0001$ and $p < 0.0001$ respectively). CSE exposure further increased VEGF release at all time points up to the maximum concentration of CSE used. IL-1β stimulation was used as a positive control (Koyama et al., 2002). At 24 hours the maximum increase of VEGF was 1.196 ± 0.05198 fold above baseline, while that at 48 hours 1.140 ± 0.07262 fold above baseline and at 72 hours 1.249 ± 0.02736 fold above baseline (Mean ± SD) (Figure 26).

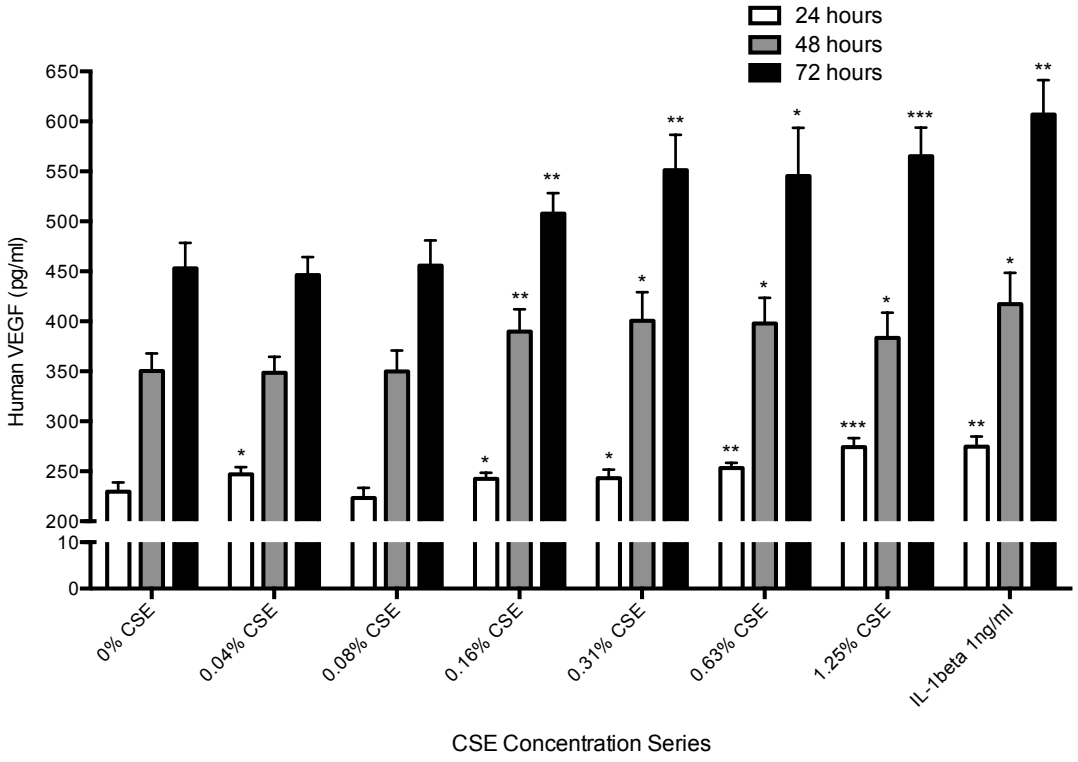

Figure 26: Influence of CSE on spontaneous VEGF production by HTEpC

HTEpC were cultured in 12 well tissue culture plates as described in section 2.4.1.1 and exposed to a concentration series of CSE. When stimulated with CSE, VEGF was released in a concentration dependent manner at 24 hours, 48 hours and 72 hours of culture (Anova $p < 0.0001$, $p < 0.0001$ and $p < 0.0001$ respectively). Paired t-test * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Mean ± SEM, n = 5

Time Course (hours)	Baseline	Concentration of Cigarette Smoke Extract						IL-1β 1 ng/ml	Anova p value
		0.04%	0.08%	0.16%	0.31%	0.63%	1.25%		
24	229.6 ± 9.262	247.1 ± 7.009 †	223.2 ± 10.21	242.6 ± 6.029 †	243.1 ± 8.630 †	253.3 ± 5.173 §	274.1 ± 9.103 ‡	274.7 ± 9.978 §	$p < 0.0001$
48	350.3 ± 17.55	348.5 ± 16.00	350.0 ± 20.82	389.7 ± 22.25 §	400.5 ± 28.56 †	397.9 ± 25.60 †	383.6 ± 25.05 †	417.2 ± 31.44 †	$p < 0.0001$
72	453.1 ± 25.51	446.3 ± 17.97	455.7 ± 25.21	507.8 ± 20.41 §	551.3 ± 35.21 §	545.4 ± 48.16 †	565.2 ± 28.65 ‡	606.8 ± 34.57 §	$p < 0.0001$

VEGF pg/ml Mean ± SEM, n = 5; Paired t-test † $p < 0.05$, § $p < 0.01$, ‡ $p < 0.001$ vs baseline

Anova comparing concentrations from baseline to 1.25% CSE

Table 7: Effect of CSE on the time course of spontaneous VEGF production by HTEpC

4.2.4 Epithelial cell interleukin-17A expression

HTEpC were cultured in 12 well tissue culture plates and exposed to a concentration series of CSE as described in section 2.4.1.1. Interleukin-17A (IL-17A) release by HTEpC was measured using ELISA (as described in section 2.5) after 24 and 72 hours of culture. The spontaneous expression of IL-17A was low and not significantly altered by CSE in the concentration range tested (Figure 27).

4.2.5 Epithelial cell interleukin-17F expression

HTEpC were cultured in 12 well tissue culture plates and exposed to a concentration series of CSE as described in section 2.4.1.1. Interleukin-17F (IL-17F) release by HTEpC was measured using ELISA (as described in section 2.5) after 24 and 72 hours of culture. The spontaneous expression of IL-17F was low and not significantly altered by CSE in the concentration range tested (Figure 28).

4.2.6 Epithelial cell thymic stromal lymphopoietin (TSLP) expression

HTEpC were cultured in 12 well tissue culture plates and exposed to a concentration series of CSE as described in section 2.4.1.1. Thymic stromal lymphopoietin (TSLP) release by HTEpC was measured using ELISA (as described in section 2.5) after 24, 48 and 72 hours of culture. HTEpC failed to release detectable TSLP after up to 72 hours of culture either spontaneously or in the presence of a concentration series of CSE as in previous experiments (data not shown).

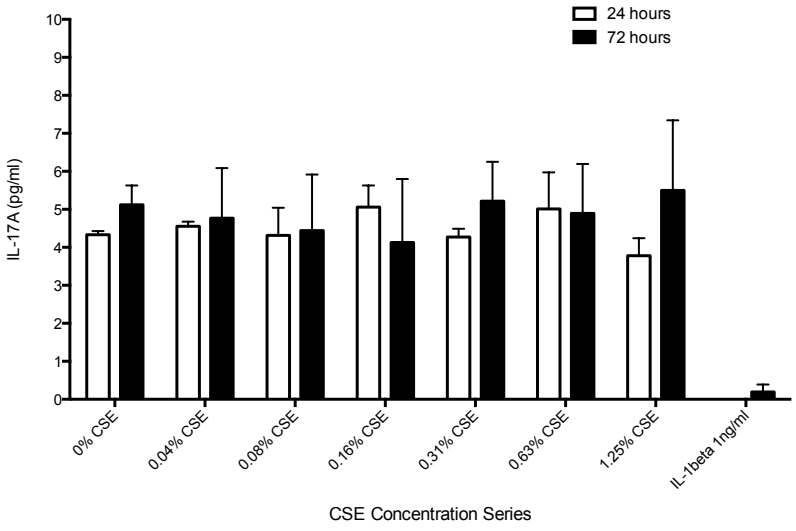

Figure 27: Influence of CSE on spontaneous IL-17A production by HTEpC

HTEpC were cultured in 12 well tissue culture plates and exposed to a concentration series of CSE, as described in section 2.4.1.1. The spontaneous expression of IL-17A was low and not significantly altered by CSE in the concentration range tested. n = 2

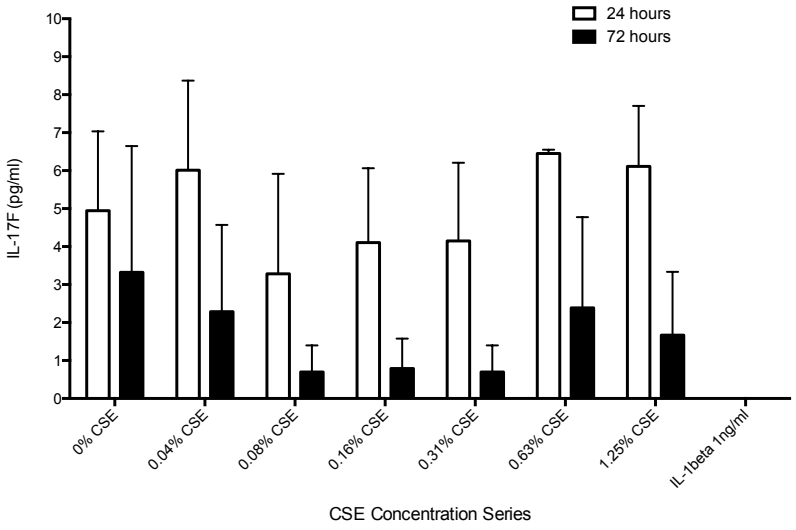

Figure 28: Influence of CSE on spontaneous IL-17F production by HTEpC

HTEpC were cultured in 12 well tissue culture plates and exposed to a concentration series of CSE, as described in section 2.4.1.1. The spontaneous expression of IL-17F was low and not significantly altered by CSE in the concentration range tested n = 2

4.3 Influence of IL-17A on CSE-induced production of pro-inflammatory and remodelling cytokines by epithelial cells

The concentration time course experiment for IL-6 and VEGF (as described in section 4.2) demonstrated that 0.16% CSE was the optimal concentration of CSE at which cellular viability was not compromised and a near maximal response to CSE was observed for the release of these cytokines. The optimal culture time for IL-6 and VEGF expression was also determined to be 24 hours. The assumption was made that the optimal culture conditions for IL-8 expression would be the same as those for IL-6 and VEGF expression. In view of this, supernatants were harvested at 24 hours to examine IL-6, IL-8 and VEGF expression.

4.3.1 Co-stimulation of human tracheal epithelial cells with cigarette smoke extract and interleukin-17A

To investigate whether bronchial epithelial cells may play a role in the maintenance of the hypothesized IL-17A mediated neutrophilic inflammation induced by cigarette smoking in asthma, experiments were conducted to test the hypothesis that cigarette smoke extract is able to enhance the pro-inflammatory and pro-remodelling cytokines produced by IL-17A stimulation of human tracheal epithelial cells. Murphy et al. had shown that IL-17A is able to increase spontaneous release of IL-6, IL-8 and VEGF in bronchial epithelial cells (Murphy et al., 2008). From the data published by Murphy et al. it was inferred that a concentration series of IL-17A of 0.1 ng/ml, 1 ng/ml and 10 ng/ml of IL-17A should be employed for the experiments investigating its effects on IL-6 and IL-8 secretion, while a concentration series of 1 ng/ml, 10 ng/ml and 100 ng/ml should be used for the experiments investigating effect on VEGF secretion.

HTEpC were cultured in 12 well tissue culture plates and were exposed to these two concentration series of IL-17A in presence or absence of 0.16% CSE, as described in section 2.4.1.2. Supernatants were harvested at 24 hours and IL-6, IL-8 and VEGF measured using ELISA (as described in section 2.5).

IL-17A alone effected a concentration dependent increase in spontaneous release of IL-6 and IL-8 by HTEpC (Anova $p < 0.0001$ and $p < 0.0001$ respectively). The additional presence of 0.16% CSE further augmented both IL-6 and IL-8 production in a concentration related, synergistic manner (Anova $p < 0.0001$ and $p = 0.0079$ respectively) (Figure 29 and Figure 30).

While IL-17A also demonstrated a concentration dependent increase in spontaneous release of VEGF by HTEpC (Anova $p < 0.0001$), the additional presence of 0.16% CSE however did not significantly alter IL-17A-induced VEGF secretion (Anova $p = 0.4028$) (Figure 31).

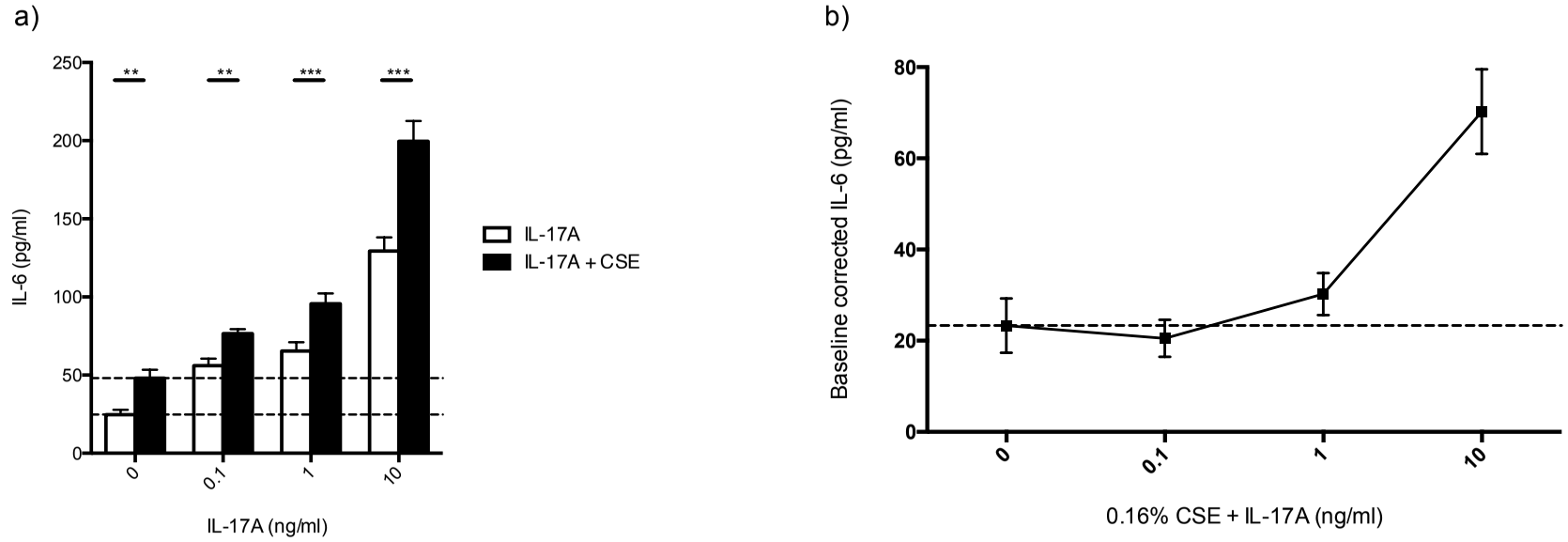

Figure 29: IL-6 production by HTEpC in response to co-stimulation with IL-17A and cigarette smoke extract

HTEpC were cultured in 12 well tissue culture plates with a concentration series of IL-17A (0, 0.1, 1 and 10 ng/ml) with or without 0.16% CSE, as described in section 2.4.1.2. a) CSE synergistically increased IL-17A-induced IL-6 secretion (IL-17A without vs with CSE, 0 µg/ml 24.71 pg/ml ± 3.075 vs 48.03 ± 5.362, $p = 0.0286$; 0.1 ng/ml 56.06 pg/ml ± 4.472 vs 76.58 ± 2.797, $p = 0.0023$; 1 ng/ml 65.36 pg/ml ± 5.632 vs 95.59 ± 6.687, $p = 0.0006$; 10 ng/ml 129.4 pg/ml ± 8.776 vs 199.6 ± 12.99, $p = 0.0003$). b) Concentration-dependent effect of IL-17A on IL-6 secretion in the presence of 0.16% CSE (Baseline 23.32 pg/ml ± 5.947; 0.1 ng/ml 20.52 pg/ml ± 4.052; 1 ng/ml 30.23 pg/ml ± 4.594; 10 ng/ml 70.25 pg/ml ± 9.290, Anova $p < 0.0001$). Paired t-test * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Mean ± SEM, $n = 7$

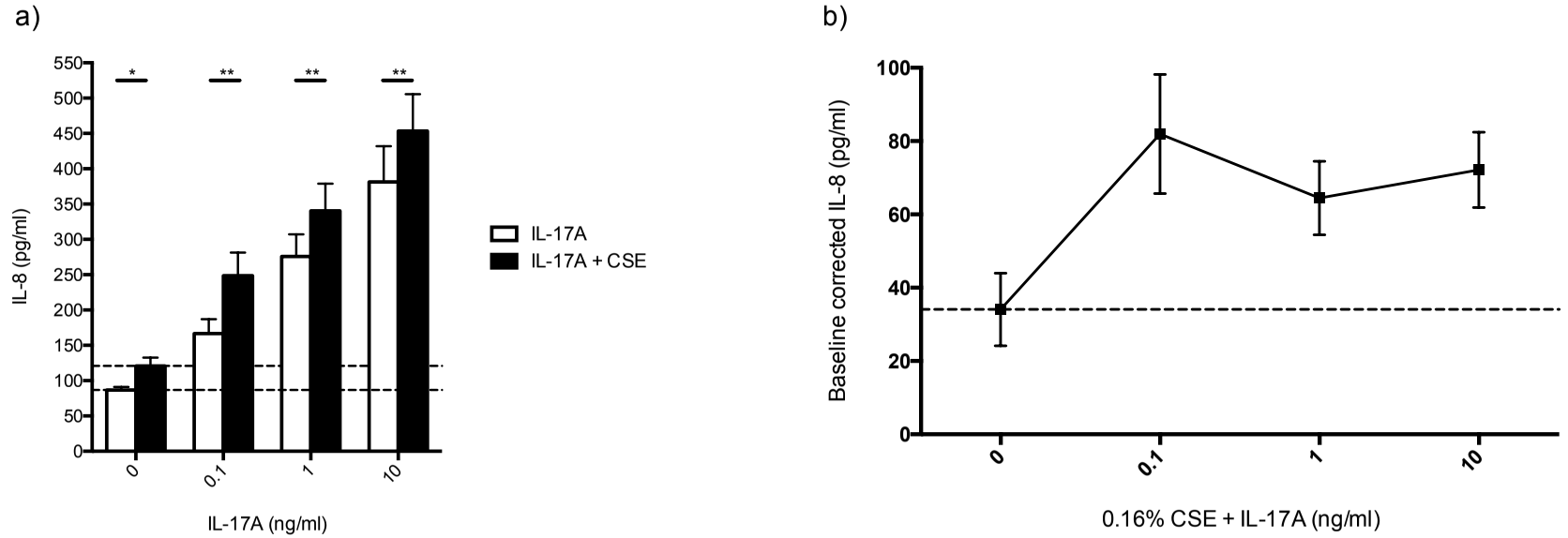

Figure 30: IL-8 production by HTEpC in response to co-stimulation with IL-17A and cigarette smoke extract

HTEpC were cultured in 12 well tissue culture plates with a concentration series of IL-17A (0, 0.1, 1 and 10 ng/ml) with or without 0.16% CSE, as described in section 2.4.1.2. a) CSE synergistically increased IL-17A-induced IL-8 secretion (IL-17A without vs with CSE, 0 µg/ml 86.73 pg/ml ± 4.335 vs 120.8 ± 11.78, p = 0.0263; 0.1 ng/ml 166.7 pg/ml ± 20.21 vs 248.7 ± 32.78, p = 0.0072; 1 ng/ml 275.9 pg/ml ± 31.33 vs 340.4 ± 38.48, p = 0.0030; 10 ng/ml 381.3 pg/ml ± 50.67 vs 453.4 ± 52.14, p = 0.0022). b) Concentration-dependent effect of IL-17A on IL-8 secretion in the presence of 0.16% CSE (Baseline 34.07 pg/ml ± 9.911; 0.1 ng/ml 81.94 pg/ml ± 16.23; 1 ng/ml 64.47 pg/ml ± 10.02; 10 µg/ml 72.16 pg/ml ± 10.29, Anova p = 0.0079). Paired t-test * $p < 0.05$, ** $p < 0.01$, Mean ± SEM, n = 5

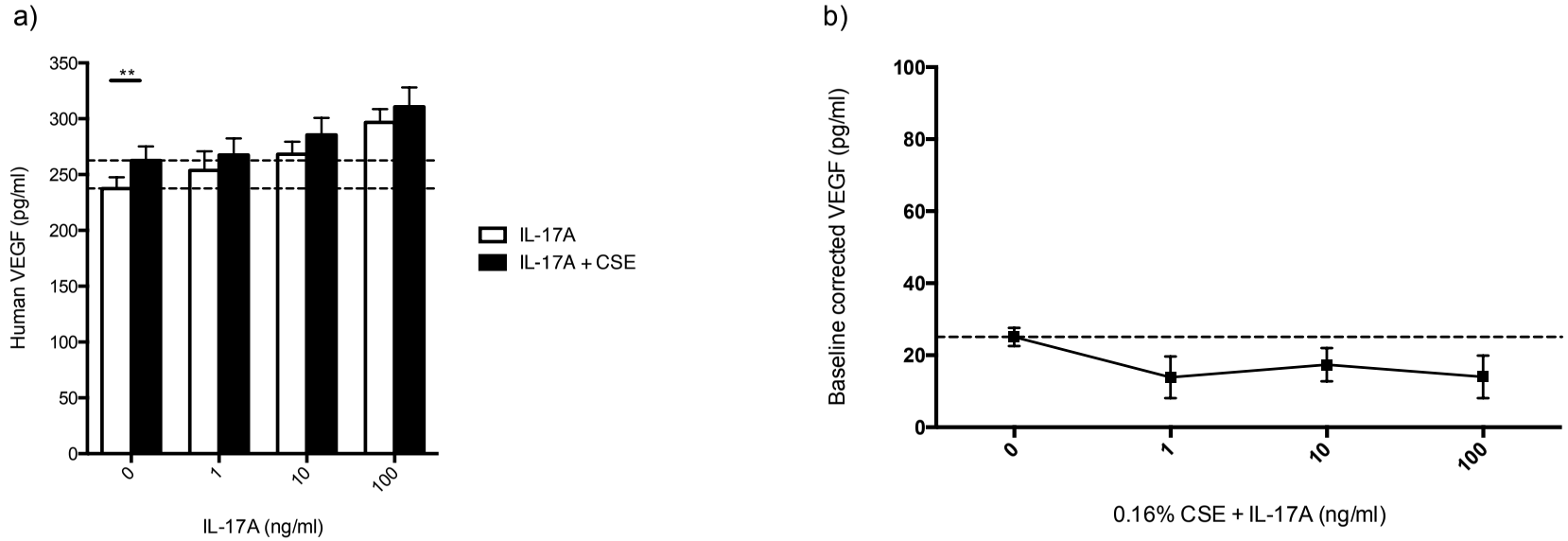

Figure 31: VEGF production by HTEpC in response to co-stimulation with IL-17A and cigarette smoke extract

HTEpC were cultured in 12 well tissue culture plates with a concentration series of IL-17A (0, 1, 10 and 100 ng/ml) with or without 0.16% CSE, as described in section 2.4.1.2. a) CSE did not significantly affect IL-17A-induced VEGF secretion (IL-17A without vs with CSE, 0 µg/ml 237.5 pg/ml ± 10.06 vs 262.6 ± 12.55, p = 0.0097; 1 ng/ml 253.6 pg/ml ± 17.27 vs 267.5 ± 14.85, p = 0.1382; 10 ng/ml 268.2 pg/ml ± 11.21 vs 285.6 ± 15.20, p = 0.0637; 100 ng/ml 296.8 pg/ml ± 11.81 vs 310.7 ± 17.24, p = 0.1419). b) The magnitude of effect of 0.16% CSE on the expression of VEGF was similar across the concentration range of IL-17A used (Baseline 25.08 pg/ml ± 2.494; 1 ng/ml 13.87 pg/ml ± 5.773; 10 ng/ml 17.39 pg/ml ± 4.613; 100 ng/ml 13.98 pg/ml ± 5.917, Anova p = 0.4028). Paired t-test ** $p < 0.01$, Mean ± SEM, n = 3

4.3.2 The effect of inhibition of reactive oxygen species formation by glutathione on co-stimulation of epithelial cells with cigarette smoke extract and interleukin-17A

It has been previously demonstrated that CSE is able to induce the generation of reactive oxygen species (ROS) by bronchial epithelial cells that in turn leads to gene transcription and release of inflammatory mediators (Kode et al., 2006, Mulligan et al., 2009). In view of this it was hypothesized that the synergistic interaction between IL-17A and CSE, as demonstrated above (section 4.3.1) in HTEpC arises at least partly from CSE generation of oxidative stress.

Glutathione (GSH) is a naturally occurring antioxidant that is present within cells. Li et al. previously demonstrated that 500 μM of GSH was able to ameliorate the effects of cigarette smoke condensate on epithelial cell permeability (Li et al., 1994). From the data published by Li et al. and our own data that 10 mM of GSH significantly effects the viability of HTEpC (data not shown), it was inferred that an optimal concentration of 1 mM of GSH, a concentration at which there was no demonstrable effect on viability, should be employed to reduce the generation of ROS. To investigate the role of oxidative stress on the synergistic interaction between IL-17A and CSE, HTEpC were cultured with 10 ng/ml IL-17A both in the presence and absence of 0.16% CSE and 1 mM of GSH (as described in section 2.4.1.3). Supernatants were harvested at 24 hours and IL-6 expression measured using ELISA, as described in section 2.5.

While GSH at a concentration of 1mM did not significantly alter IL-6 expression induced by 0.16% CSE in HTEpC ($p = 0.7173$), it did significantly reduce IL-6 production induced by both IL-17A ($p = 0.0043$) and by co-stimulation with CSE and IL-17A ($p = 0.0211$) (Figure 32).

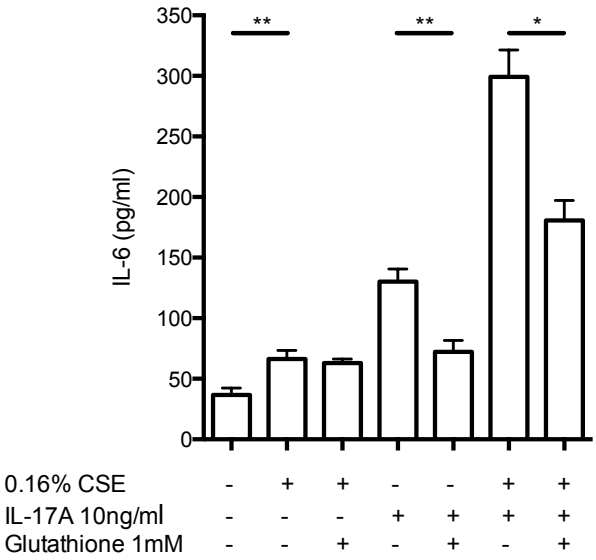

Figure 32: Effect of glutathione on IL-6 production by HTEpC co-stimulated with IL-17A and cigarette smoke extract

HTEpC were cultured in 12 well tissue culture plates with 0.16% CSE and/or IL-17A with or without 1 mM of Glutathione (GSH) as described in section 2.4.1.3. CSE significantly increased the expression of IL-6 compared to baseline ($p = 0.0045$). There was no significant inhibition of CSE induced expression of IL-6 by 1 mM of GSH ($p = 0.7173$). However 1 mM of GSH significantly inhibited the expression of IL-6 induced by IL-17A alone and in combination with CSE ($p = 0.0043$ and $p = 0.0211$ respectively). Paired t-test * $p < 0.05$, ** $p < 0.01$, Mean ± SEM, n = 4

4.3.3 The effect of aeroallergens on co-stimulation of epithelial cells with cigarette smoke extract and interleukin-17A

Bronchial epithelial cells not only function as a physical barrier to aeroallergens but are also able to release pro-inflammatory mediators in response to exposure to a variety of aeroallergens (Bhat et al., 2003, Pichavant et al., 2005, Tai et al., 2006, Osterlund et al., 2009, Leino et al., 2013). In view of the fact that CSE and IL-17A were able to interact with HTEpC in a synergistic manner to release pro-inflammatory mediators (section 4.3.1) it was hypothesised that the aeroallergens cat dander and timothy grass pollen are able to influence this interaction.

Whole allergen extracts, which were adjuvant free and used for subcutaneous immunotherapy, were employed for these experiments. The allergen extracts were Aquagen SQ manufactured by Alk Abello, with a stock concentration of 100,000 SQ-U/ml. These were used a concentration of 5000 SQ-U/ml of allergen as preliminary unpublished data from another group within the division, had indicated that there was an interaction between 5000 SQ-U/ml of allergen and diesel exhaust particle stimulation of epithelial cells.

HTEpC were cultured in 12 well tissue culture plates and exposed to 0.16% CSE and/or 10 ng/ml IL-17A both in the presence and absence of 5000 SQ-U/ml of cat dander or timothy grass pollen, as described in section 2.4.1.4. Supernatants were harvested at 24 hours and IL-6 and IL-8 expression measured using ELISA as described in section 2.5.

4.3.3.1 Cat dander aeroallergen

Spontaneous secretion of IL-6 by the tracheal epithelial cells was significantly increased by 0.16% CSE, 10 ng/ml IL-17A and cat dander extract at 5000 SQ-U/ml ($p = 0.0086$, $p = 0.0095$ and $p = 0.0062$ respectively) (Figure 33 a). While cat dander did not significantly enhance the effects of CSE or IL-17A alone ($p = 0.0594$ and $p = 0.2676$ respectively), it did further significantly increase IL-6 production by the combination ($p = 0.0004$) (Figure 33 a).

Similarly, spontaneous secretion of IL-8 by the tracheal epithelial cells was significantly increased by 0.16% CSE, 10 ng/ml IL-17A and cat dander extract at 5000 SQ-U/ml ($p < 0.0001$, $p = 0.0002$ and $p = 0.0109$ respectively) (Figure 33 b). While cat dander did not significantly enhance the effects of CSE or IL-17A alone ($p = 0.9648$ and $p = 0.1573$ respectively), it did further significantly increase IL-8 production by the combination ($p = 0.0340$) (Figure 33 b).

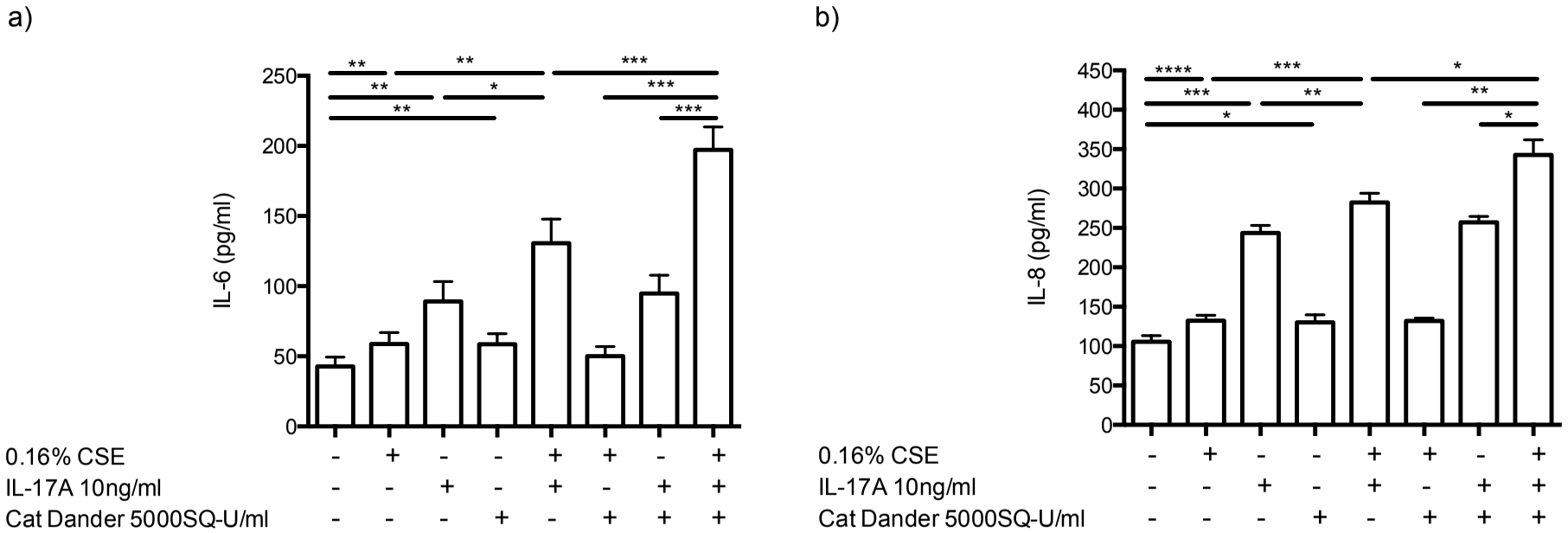

Figure 33: Effect of cat dander on co-stimulation of HTEpC with IL-17A and cigarette smoke extract

HTEpC were cultured in 12 well tissue culture plates and exposed to 0.16% CSE and/or 10 ng/ml IL-17A both in the presence and absence of 5000 SQ-U/ml of cat dander as described in section 2.4.1.4. Supernatants were harvested at 24 hours and IL-6 and IL-8 expression measured using ELISA as described in section 2.5. a) Cat dander further significantly increased the expression of IL-6 induced by co-simulation with CSE and IL-17A (130.7 pg/ml ± 17.12 vs 197.2 pg/ml ± 16.32, $p = 0.0004$). b) Cat dander similarly further significantly increased the expression of IL-8 induced by co-simulation with CSE and IL-17A (282.2 pg/ml ± 11.71 vs 342.7 pg/ml ± 19.30, $p = 0.0340$). Paired t-test * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Mean ± SEM, n = 4

4.3.3.2 Timothy grass pollen aeroallergen

Spontaneous secretion of IL-6 by the tracheal epithelial cells was significantly increased by 0.16% CSE, 10 ng/ml IL-17A and timothy grass pollen extract at 5000 SQ-U/ml ($p = 0.0086$, $p = 0.0038$ and $p = 0.0042$ respectively) (Figure 34 a). While timothy grass pollen did not significantly enhance the effects of CSE or IL-17A alone ($p = 0.1699$ and $p = 0.9770$ respectively), it did further significantly increase IL-6 production by the combination ($p = 0.0084$) (Figure 34 a).

Similarly, spontaneous secretion of IL-8 by the tracheal epithelial cells was significantly increased by 0.16% CSE, 10 ng/ml IL-17A and timothy grass pollen extract at 5000 SQ-U/ml ($p < 0.0001$, $p < 0.0001$and $p = 0.0098$ respectively) (Figure 34 b). While timothy grass pollen did not significantly enhance the effects of CSE or IL-17A alone ($p = 0.2791$ and $p = 0.1861$ respectively), it did further significantly increase IL-6 production by the combination ($p = 0.0114$) (Figure 34 b).

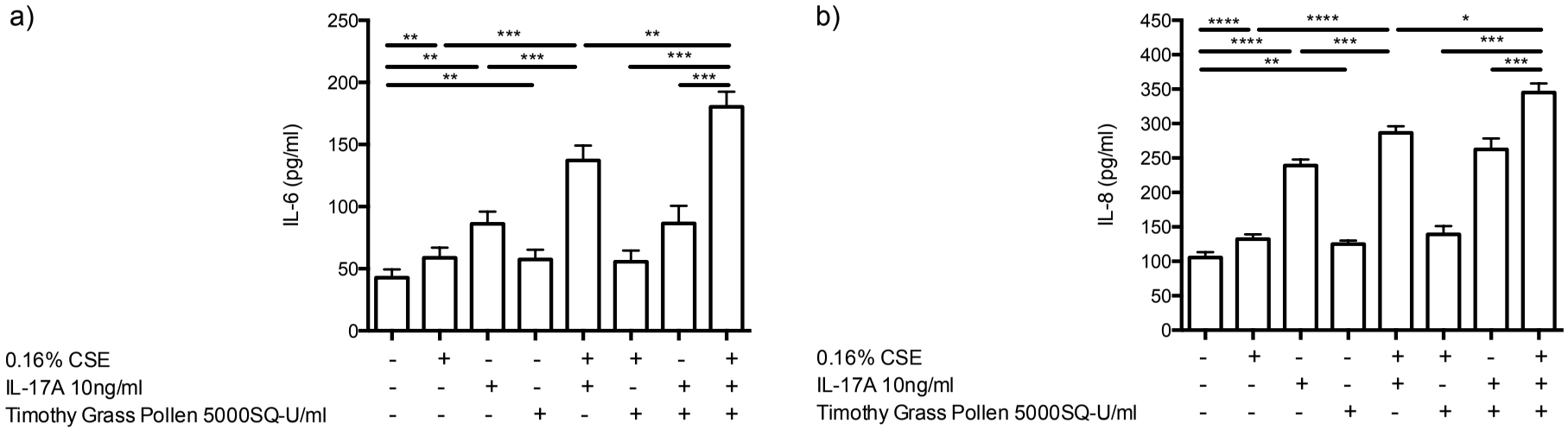

Figure 34: Effect of timothy grass pollen on co-stimulation of HTEpC with IL-17A and cigarette smoke extract

HTEpC were cultured in 12 well tissue culture plates and exposed to 0.16% CSE and/or 10 ng/ml IL-17A both in the presence and absence of 5000 SQ-U/ml of timothy grass pollen as described in section 2.4.1.4. Supernatants were harvested at 24 hours and IL-6 and IL-8 expression was measured using ELISA as described in section 2.5. a) Timothy grass pollen further significantly increased the expression of IL-6 induced by co-simulation with CSE and IL-17A (137.2 pg/ml ± 12.05 vs 180.4 pg/ml ± 12.17, $p = 0.0084$). b) Timothy grass pollen similarly further significantly increased the expression of IL-8 induced by co-simulation with CSE and IL-17A (286.6 pg/ml ± 9.543 vs 345.2 pg/ml ± 13.09, $p = 0.0114$). Paired t-test * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Mean ± SEM, $n = 4$

4.4 Effects of cigarette smoke extract on production of inflammatory mediators by epithelial cells in response to a Toll-like receptor 3 agonist

Thymic stromal lymphopoietin (TSLP) has been shown to play an important role in the generation of Th2 mediated inflammation in asthma (Soumelis et al., 2002, Ying et al., 2005). The activation of Toll-like receptor 3 (TLR-3), a pattern recognition receptor which recognises non-methylated viral nucleic acids, has been shown to be able to induce the expression of TSLP in bronchial epithelial cells (Kato et al., 2007). Furthermore viral infections are a known exacerbating factor for asthma (Dougherty and Fahy, 2009). Consequently we investigated the ability of CSE to influence expression of TSLP and IL-6 induced by TLR-3 activation. We induced the activation of TLR-3 using a synthetic surrogate of double-stranded viral RNA (dsRNA), Polyinosinic:polycytidylic acid (Poly I:C). From the data published by Kato et al. (Kato et al., 2007) it was inferred that a concentration series of 0.1 μg/ml, 1 μg/ml and 10 μg/ml of Poly I:C was optimal to induce the expression of TSLP, and a 24 hour culture period sufficient. In view of this HTEpC were cultured in 12 well tissue culture plates and exposed to this concentration series of Poly I:C with or without the presence of 0.16% CSE (the concentration of CSE which was shown above in section 4.3.1 to interact synergistically with IL-17A), as described in section 2.4.1.5. Supernatants were harvested at 24 hours and TSLP and IL-6 expression measured using ELISA as described in section 2.5. Vehicle controls were not used in this experiment as Poly I:C was reconstituted in sterile, endotoxin-free physiological water (NaCl 0.9%), as supplied and in accordance with the manufacturer's instructions.

Spontaneous secretion of TSLP by epithelial cells in the presence and absence of 0.16% CSE was undetectable. Poly I:C induced epithelial cells to release TSLP in a concentration dependent manner (Anova $p < 0.0001$) and interestingly this was significantly diminished in the presence of 0.16% CSE (Poly I:C without CSE vs. with CSE; 0.1 μg/ml 123.5 pg/ml ± 10.50 vs 102.4 pg/ml ± 5.501, $p = 0.0412$; 1 μg/ml 166.1 pg/ml ± 11.05 vs 142.3 pg/ml ± 7.641, $p = 0.0135$; and 10 μg/ml 174.7 pg/ml ± 13.14 vs 148.4 pg/ml ± 11.89, $p = 0.0036$) (Figure 35 a).

Spontaneous secretion of IL-6 was 20.60 pg/ml ± 4.226 in the absence of 0.16% CSE and 36.27 pg/ml ± 7.986 in the presence of 0.16% CSE ($p = 0.0286$). Poly I:C increased spontaneous secretion of IL-6 in a concentration dependent manner (Anova $p < 0.0001$) and this was not significantly altered in the presence of 0.16% CSE (Poly I:C without CSE vs. with CSE 0.1 μg/ml 1393 pg/ml ± 83.23 vs 1386 pg/ml ± 23.67, $p = 0.9278$; 1 μg/ml 1564 pg/ml ± 50.62 vs 1674 pg/ml ± 4.937, $p = 0.1350$; and 10 μg/ml 1713 pg/ml ± 33.33 vs 1711 pg/ml ± 51.26, $p = 0.9754$) (Figure 35 b).

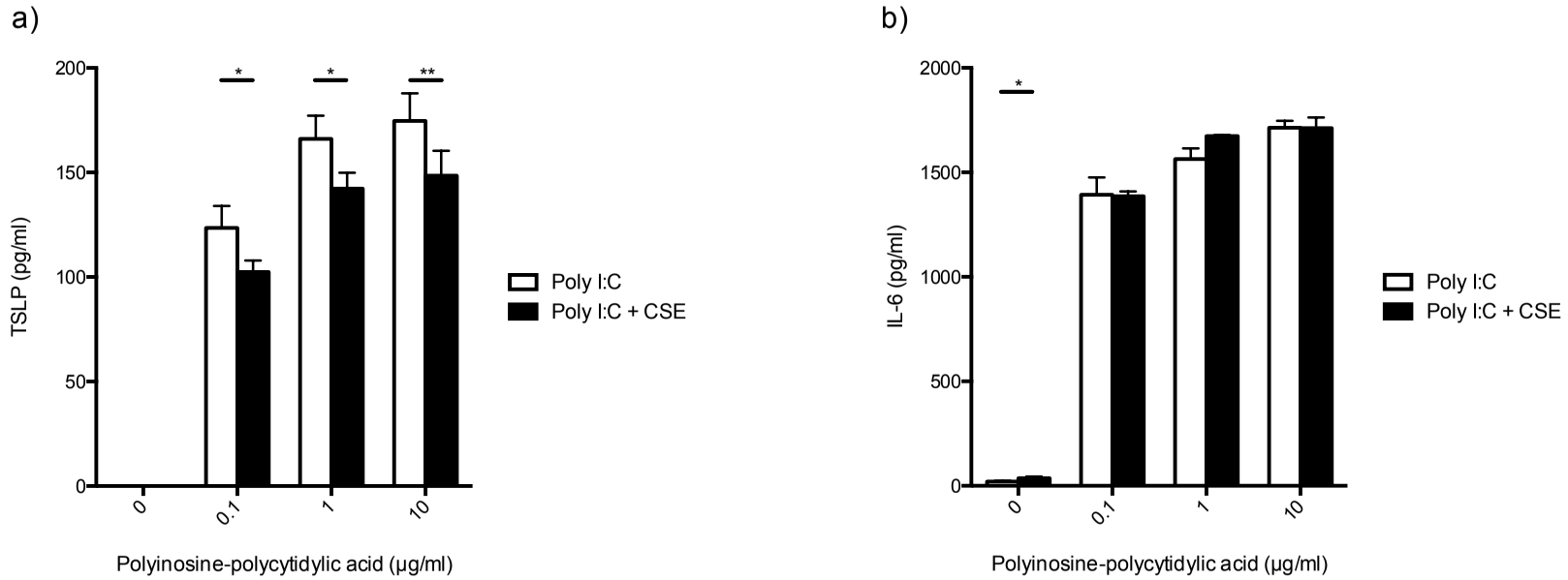

Figure 35: Effects of co-stimulation of HTEpC with polyinosinic:polycytidylic acid (Poly I:C) and cigarette smoke extract

HTEpC were cultured in 12 well tissue culture plates and exposed to 0.1 µg/ml, 1 µg/ml and 10 µg/ml of Poly I:C in the presence and absence of 0.16% CSE as described in section 2.4.1.5. Supernatants were harvested at 24 hour and TSLP and IL-6 expression measured using ELISA as described in section 2.5. a) Spontaneous secretion of TSLP by epithelial cells in the presence and absence of 0.16% CSE was undetectable. Poly I:C induced epithelial cells to release TSLP in a concentration dependent manner (Anova $p < 0.0001$) and interestingly this was significantly diminished in the presence of 0.16% CSE. b) Poly I:C increased spontaneous secretion of IL-6 in a concentration dependent manner (Anova $p < 0.0001$) and this was not significantly altered in the presence of 0.16% CSE. Paired t-test * $p < 0.05$, ** $p < 0.01$, Mean ± SEM, $n = 4$

4.5 Signal transduction pathway of cigarette smoke extract - induced expression of VEGF in airways epithelial cells

To investigate the signalling pathway by which CSE induces the expression of VEGF in HTEpC, HTEpC were cultured with CSE and various intracellular inhibitors to address the possible involvement of the Nuclear Factor Kappa-light-chain-enhancer of activated B cells (NF-κB) signalling pathway, Phosphatidylinositide 3-kinase (PI3K) signalling pathway and Mitogen-activated protein (MAP) Kinase signalling pathways.

The NF-κB signalling pathway was inhibited using the intracellular inhibitor BAY 11-7082. HTEpC were exposed to 0.16% CSE and 2.5μM BAY 11-7082 (Lee et al., 2007), as described in section 2.4.1.6.

The MAPK/Erk signalling pathway was inhibited using the intracellular inhibitors PD 98059 (MEK1 inhibitor) and U 0126 (MEK1/2 inhibitor). HTEpC were cultured with 0.16% CSE and 5μM PD 98059 (Cheung et al., 2005), and 0.16% CSE and 10μM U 0126 (Monick et al., 2005), as described in section 2.4.1.6.

The MAPK/p38 MAPK signalling pathway was inhibited using the intracellular inhibitor SB 203580. HTEpC were cultured with 0.16% CSE and 5μM SB 203580 (Takahashi et al., 2007), as described in section 2.4.1.6.

The MAPK/JNK signalling pathway was inhibited using the intracellular inhibitor SP 600125. HTEpC were cultured with 0.16% CSE and 2.5μM SP 600125 (Ju et al., 2002), as described in section 2.4.1.6.

The involvement of PI3 kinase signalling was investigated using the intracellular inhibitor LY 294002. HTEpC were cultured with 0.16% CSE and 10μM LY 294002 (Kamata et al., 2004), as described in section 2.4.1.6.

HTEpC were cultured in 12 well tissue culture plates with 0.16% CSE and the intracellular inhibitors above as described in section 2.4.1.6. For baseline control

expression, HTEpC were cultured with and without 0.16% CSE. Vehicle controls were DMSO in medium diluted to the same dilution factor used for the various intracellular inhibitors (i.e. 1/500, 1/1000, 1/2000, 1/40000). The cells were cultured for 24 hours and supernatants harvested for VEGF measurement using ELISA.

The baseline expression of VEGF was 335.7 pg/ml ± 28.45 in the absence of 0.16% CSE and 404.2 pg/ml ± 29.13 in the presence of 0.16% CSE. SB 203580, PD 98059, U 0126, SP 600125 and LY 294002 inhibited both spontaneous and CSE-induced expression of VEGF (213.9 pg/ml ± 16.39, $p = 0.0012$; 228.2 pg/ml ± 11.66, $p = 0.0033$, 151.8 pg/ml ± 11.26, $p = 0.0010$, 308.3 pg/ml ± 19.82, $p = 0.0068$, 274.5 pg/ml ± 16.61, $p = 0.0042$ respectively). BAY 11-7082 however had no effect on the expression of VEGF induced by 0.16% CSE (372.6 pg/ml ± 28.63, $p = 0.1089$). Vehicle control did not affect on the baseline expression of VEGF (DMSO 1/500 dilution 333.3 pg/ml ± 24.95, $p = 0.8413$) (Figure 36).

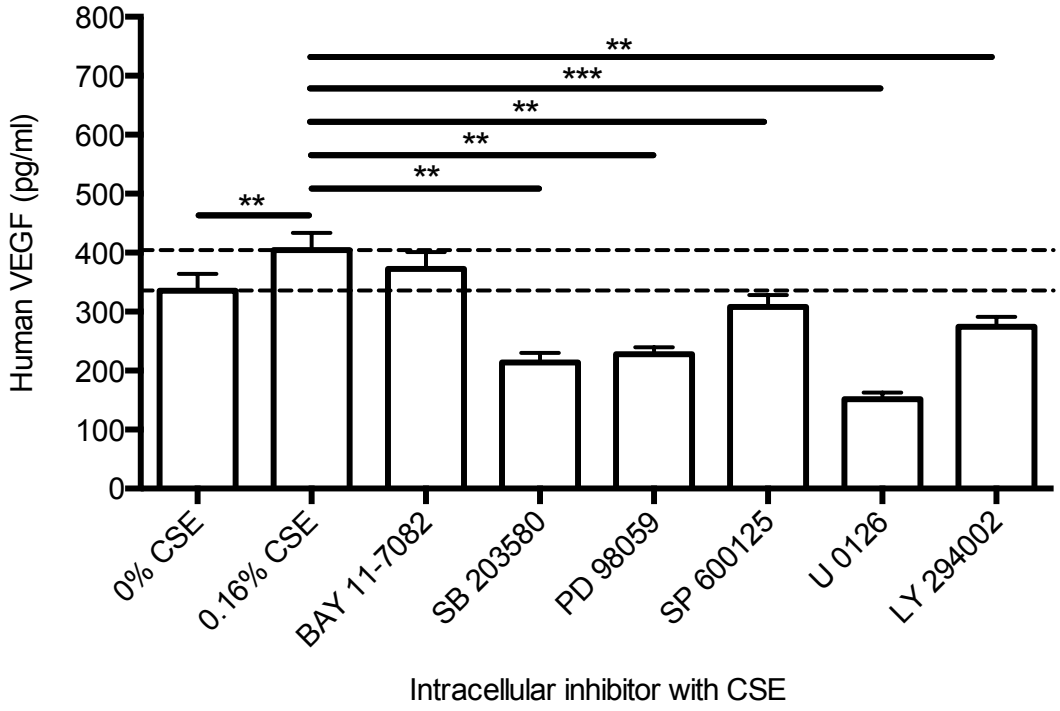

Figure 36: Effect of signalling transduction inhibitors on the spontaneous and CSE-induced production of vascular endothelial growth factor by HTEpC

HTEpC were cultured in 12 well tissue culture plates with 0.16% CSE and various intracellular inhibitors as described in section 2.4.1.6. 0.16% CSE significantly increased the expression of VEGF (0% CSE 335.7 pg/ml ± 28.45 vs 0.16% CSE 404.2 pg/ml ± 29.13, $p = 0.0034$). Both spontaneous and CSE-induced expression of VEGF was inhibited by SB 203580, PD 98059, U 0126, SP 600125 and LY 294002 (213.9 pg/ml ± 16.39, $p = 0.0012$; 228.2 pg/ml ± 11.66, $p = 0.0033$, 151.8 pg/ml ± 11.26, $p = 0.0010$, 308.3 pg/ml ± 19.82, $p = 0.0068$, 274.5 pg/ml ± 16.61, $p = 0.0042$ respectively). BAY 11-7082 however had no effect on the expression of VEGF induced by 0.16% CSE (372.6 pg/ml ± 28.63, $p = 0.1089$). Vehicle control did not affect the baseline expression of VEGF (DMSO 1/500 dilution 333.3 pg/ml ± 24.95, $p = 0.8413$). Paired t-test ** $p < 0.01$, *** $p < 0.001$, Mean ± SEM, $n = 4$

4.6 Discussion and summary

HTEpC were exposed to a concentration series of CSE (0 – 2.5% CSE) it was verified that this concentration range of CSE did not affect the viability of the cells.

Exposure of HTEpC to cigarette smoke extract (CSE) significantly increased their expression of IL-6 and IL-8. This is consistent with what has been previously described by both Wyatt et al. and Kode et al. (Wyatt et al., 1999, Kode et al., 2006). Furthermore, it was demonstrated that CSE is able to induce the expression of VEGF in HTEpC in a concentration dependent manner. CSE however did not induce the expression of IL-17A, IL-17F or TSLP in HTEpC.

During the course of this body of work, Heijink et al. in 2013 published data suggesting that CSE is able to induce a modest expression of VEGF in 16HBE cells and that this expression was augmented by co-stimulation with recombinant human WNT-4 (wingless-type MMTV integration site family, member 4; a secreted signalling protein which has been implicated to play a role in lung epithelial repair process) (Crosby and Waters, 2010, Heijink et al., 2013). The present data further advance their data by showing that the induction of VEGF by CSE is dependent on the 3 main MAP Kinase signalling molecules (p38 MAPK, Erk and JNK) and, upstream from this, the PI3 kinase-dependent Akt phosphorylation pathway. The involvement of all 3 main MAP Kinase signalling pathways in VEGF induction by CSE is similar to that seen in IL-31 induction of VEGF in BEAS-2B cells, which was also shown to be dependent on p38 MAPK, Erk1 and JNK1/2 (Ip et al., 2007).

In contrast the present data and those of Heijink et al., Volpi et al. reported that cigarette smoke did not induce expression of VEGF in small airways epithelial cells (SAEC) (Volpi et al., 2011). Furthermore, Thaikoottathil et al. had reported that CSE inhibited the expression of VEGF in both SEAC and normal human bronchial epithelial cells (NHBE) following single and repeated exposure to CSE over 1 to 14 days (Thaikoottathil et al., 2009).

A possible explanation for these decrepancies is that the concentrations of CSE with which both Volpi and Thaikoottahil were working were towards the top end of a skewed, bell shaped concentration response curve similar to that observed for CSE-induced IL-6 production, demonstrated in this thesis. Where lower concentrations of CSE might induce VEGF in airways epithelial cells, higher concentrations might reduce it. Given that different techniques were used in both the preparation of CSE and the method of exposure to CSE, it is difficult directly to compare the exact exposure to CSE in these various publications.

The present findings are consistent with those published by Murphy et al. in 2008 showing that IL-17A induces the expression of IL-6, IL-8 and VEGF in primary bronchial epithelial cells. They also showed that IL-17A induces the expression of granulocyte colony-stimulating factor and granulocyte-macrophage colony-stimulating factor (Murphy et al., 2008). We have extended these findings by demonstrating that CSE is able to augment the expression of both IL-6 and IL-8 induced by IL-17A in a synergistic manner. In contrast, CSE had no effect on IL-17A-induced VEGF expression. This would imply that CSE is able to support and augment IL-17A mediated inflammation of the airways.

We investigated the role of reactive oxygen species (ROS) in the interaction between IL-17A and CSE. Glutathione was able significantly to inhibit the release of IL-6 induced by IL-17A alone and in combination with CSE. This would suggest that the induction of IL-6 by IL-17A in HTEpC is at least partly mediated by the generation of ROS by IL-17A. Interestingly the concentration of glutathione employed in these studies had no significant effect on CSE induced IL-6 expression. Possible explanations for this are that the amount of ROS generated by 0.16% CSE is significantly higher than that generated by IL-17A so that the concentration of glutathione employed did not sufficiently neutralise it, or that IL-6 induction in HTEpC by CSE does not involve ROS.

Montalbano et al. recently demonstrated that both CSE and IL-17A independently increase ROS production by 16HBE epithelial cells. Furthermore an additive increase in ROS was seen when 16HBE cells were co-stimulated with CSE and IL-17A

(Montalbano et al., 2013). It is therefore possible to hypothesise that the mechanism by which CSE and IL-17A interact to increase the release of the pro-inflammatory cytokines IL-6 and IL-8 by HTEpC involves the generation of ROS.

Inhaled aeroallergens are thought to be a significant drive to airways allergic inflammation in asthma. Furthermore the bronchial epithelium, as a structural barrier, is the primary site where both inhaled aeroallergens and cigarette smoke make contact with the body. It is therefore plausible that an interaction between cigarette smoke and inhaled allergens could occur at this juncture. The data here presented suggest that both cat dander and timothy grass pollen aeroallergens are able further to augment the expression of IL-6 and IL-8 when HTEpC are co-stimulated with both IL-17A and CSE, but not with either stimulus alone. Tomee et al. showed that both timothy grass pollen and birch pollen lack protease activity (as quantified by the ability to hydrolyse AZO-bovine serum albumin) and were able to induce the expression of IL-6 and IL-8 in A549 epithelial cells despite heat treatment (Tomee et al., 1998). Their findings were corroborated by Röschmann et al., who advanced them by showing that the p38 MAPK and Erk signalling pathways are involved in Timothy grass pollen major allergen Phl p1 induction of IL-6 and IL-8 expression in A549 cells (Roschmann et al., 2009). Furthermore Osterlund et al. showed that both natural and recombinant Fel d1 allergen was able to induce the expression of GM-CSF, IL-6, IL-8, MCP-1 and MIP-3 in BEAS-2B cells (Osterlund et al., 2011). The mechanisms by which non-protease allergens are able to induce bronchial epithelial cells to release pro-inflammatory cytokines is not well characterised. In view of the fact that both cat dander and timothy grass pollen mediate their effects in bronchial epithelial cells in a protease independent manner, one might hypothesise that the synergistic effect seen here with IL-17A, CSE and allergen is also likely to be due to a protease independent mechanism. We believe that this is the first time synergistic interactions between IL-17A, CSE and allergen to induce the expression of pro-inflammatory cytokines have been demonstrated in bronchial epithelial cells.

Rusznak et al. showed that brief exposure of human bronchial epithelial cells (HBEC) cigarette smoke inhibited the release of IL-8, while increasing the release of IL-1β when HBEC were stimulated with house dust mite extract. On prolonged exposure to

cigarette smoke there was a reduction in the spontaneous release and house dust mite induced release of IL-8 and IL-1β (Rusznak et al., 2001). In contrast, the present studies revealed no difference in the expression of both IL-6 and IL-8 by HTEpC when exposed to 0.16% CSE alone or in combination with cat dander/timothy grass pollen. The mechanism by which cat dander and timothy grass pollen exert their pro-inflammatory effects on bronchial epithelial cells is thought to be via a protease independent mechanism (Tomee et al., 1998, Roschmann et al., 2009, Osterlund et al., 2011), whereas house dust mite exerts its effects through both protease dependent and independent mechanisms (King et al., 1998, Kauffman et al., 2006, Osterlund et al., 2009). Varying mechanisms of action of protease and non-protease allergens could partly account for this discrepancy.

In normal bronchial epithelial cells, CSE is able to inhibit viral infection-induced expression of CXCL10/IP-10, CCL5, IFN-β, antiviral proteins (viperin, ISG15 and ISG56), while increasing viral replication and synergistically increasing the expression of IL-8 and CCL2/MCP-1 (Castro et al., 2008, Hudy et al., 2010, Eddleston et al., 2011, Proud et al., 2012). Furthermore in BEAS-2B cells CSE has been shown to inhibit TLR-3 induced expression of type I interferons (Bauer et al., 2008), while in primary nasal epithelial cells CSE has been shown synergistically to increase Poly I:C induced expression of IL-1β, IL-6 and IL-8 mRNA (Yamin et al., 2008). The present study extends these findings by showing that CSE is able significantly to inhibit TLR-3 mediated TSLP expression but not IL-6 expression.

Horvath et al. utilised a monocyte-derived dendritic cell, nasal epithelial cell co-culture system to investigate the effect of cigarette smoking on the epithelial/dendritic cell response to influenza A virus infection. They found that nasal epithelial cells (NEC) harvested from smoking healthy volunteers compared to those from non-smokers showed increased TSLP expression and reduced CXCL10/IP-10 and RANTES expression following influenza A virus infection. Of note their data did not show that an increase in TSLP was observed following influenza infection in NEC obtained from non-smokers (Horvath et al., 2011). This is surprising considering that influenza A virus has been shown to activate TLR-3 in lung epithelial cells (Le Goffic et al., 2007) and it is well described that TLR-3 activation leads to the expression of

TSLP (Kato et al., 2007). Furthermore evidence from animal models suggests that TSLP increases following influenza A infection (Yadava et al., 2013). The discrepancy between the present findings and those of Hovarth et al. might be explained by acute affects of CSE causing suppression of TSLP expression, and subsequently the response seen by Hovarth et al. could represent a "rebound" response following direct suppression by CSE. Furthermore the response they described might be a non-TLR-3 mediated response since influenza A virus is a negative-sense single-stranded RNA (Group V) virus which could activate TLR-7, TLR-8 and RIG-1 as well as TLR-3 (Boehme and Compton, 2004, Xagorari and Chlichlia, 2008).

In summary we have demonstrated that CSE is able to support and promote the production of pro-inflammatory and pro-remodelling cytokines induced by IL-17A in bronchial epithelial cells and that inhaled aeroallergens have the potential further to augment the interaction between CSE and IL-17A. While supporting Th17/IL-17A mediated inflammation, CSE is also able to inhibit the release of TSLP, an important cytokine in the development of Th2 mediated inflammation in asthma.

Chapter 5: Endobronchial biopsies: *in vivo* experiments

5 Endobronchial biopsies: *in vivo* experiments

5.1 Introduction

Cadaveric lungs and endobronchial biopsies from asthmatic patients have aided in the study of the pathogenesis of asthma. These studies have shed light on both the inflammatory infiltration profile in the airways of asthmatics as well as identifying characteristic changes in the airways that have been termed "remodelling".

5.1.1 Vascular remodelling in asthma

In the bronchial mucosa of asthmatic patients there is an increase in the expression of vascular endothelial growth factor (VEGF). VEGF is a major angiogenic growth factor and its over expression in the airways of asthmatics results in increased vascularity of the airways, as evidenced by an increase in the number of blood vessels as vascular area in the bronchial mucosa (Chetta et al., 2005, Feltis et al., 2006). Increased vascularity and vascular leakiness assist the infiltration of inflammatory cells into the bronchial mucosa (Khor et al., 2009). The vascular "remodelling" seen in the airways of asthmatic patients may be reduced by the treatment with inhaled corticosteroids (Hoshino et al., 2001b, Feltis et al., 2007).

5.1.2 Infiltration of inflammatory cells in asthma

The inflammatory profile in asthma is characterised by the influx of eosinophils and Th2 cells (Robinson et al., 1993, Ying et al., 1995). It has been long thought that the allergic inflammation seen in asthma is orchestrated primarily by Th2 cells and this results in the characteristic inflammatory cellular infiltration and squealae of airways remodelling seen in the bronchial mucosa of asthmatic patients (Macedo et al., 2009, Martinez and Vercelli, 2013). While this pattern of inflammation is seen primarily in the airways of mild to moderate asthmatic patients, in the airways of severe asthmatic patients there is also an influx of neutrophils into the bronchial mucosa (Wenzel et al., 1999). The mechanism by which airway neutrophilia develops in severe asthmatics is poorly understood and there is an interesting prevailing dichotomy "is the neutrophilia a result of uncontrolled eosinophilic inflammation or is it due to chronic treatment with corticosteroids". Recent developments in this field have provided evidence to support the involvement of Th17 cells in airways inflammation of asthma (Shi et al.,

2011, Nanzer et al., 2013). Th17 cells are characterised by the release of IL-17A, which in turn stimulates structural cells to release the neutrophil chemoattactant IL-8 (Laan et al., 1999, Molet et al., 2001, Vanaudenaerde et al., 2003, Ivanov et al., 2006).

5.1.3 Effects of cigarette smoke exposure on airways remodelling and inflammation

Studies using endobronchial biopsies have suggested that the principal difference in remodelling changes in the airways of asthmatic smokers compared to non-smokers is an increase in epithelial "remodelling" of the airways, with no significant difference in the degree of airway smooth muscle hypertrophy/hyperplasia or reticular basement membrane thickening. The bronchial mucosa of mild asthmatic smokers typically shows neutrophilic infiltration, which is not usually seen in mild asthmatic non-smokers but is more frequently found in severe asthma (St-Laurent et al., 2008, Broekema et al., 2009). We are not aware of any publications that have compared the degree of vascular "remodelling" in mild asthmatic smokers compared to non-smokers.

5.1.4 Summary

There is paucity of data to show that there is increased airways "remodelling" in the bronchial mucosa of mild asthmatic smokers, despite the presence of a cellular inflammatory profile that is associated with severe asthma previous data presented in this thesis (Chapters 3 and 4) have shown that cigarette smoke extract (CSE) is able to increase the spontaneous expression of VEGF, IL-6 and TGF-β1 by airways structural cells (fibroblasts and epithelial cells). Furthermore, these new data show that CSE is able further to augment the expression of both pro-inflammatory (IL-6 and IL-8) and pro-remodelling (VEGF) cytokines. Both IL-6 and TGF-β1 are key cytokines required for the differentiation of naïve T cells to Th17 cells (Ivanov et al., 2006).

The overarching hypothesis addressed in this thesis is that cigarette smoking promotes an increase in vascular "remodelling" and the development of a Th17/IL-17A mediated inflammatory response in asthmatic smokers. It is further hypothesised that the neutrophilic inflammation seen in smoking athmatics is mediated by IL-17A, the

characteristic cytokine of Th17 cells (Laan et al., 1999, Molet et al., 2001, Vanaudenaerde et al., 2003, Ivanov et al., 2006).

The main objective of the studies described in this chapter is to investigate the expression of VEGF and degree of vascular "remodelling", as well as the expression of pro-inflammatory cytokines associated with Th17 inflammation in the bronchial mucosa of mild asthmatic smokers compared to non-smokers. To test this hypothesis, immunohistochemical studies were performed on endobronchial biopsies harvested from smoking and non-smoking asthmatics. The following markers were examined:

Neutrophilia hypothesis

1. IL-8 (the main neutrophil chemoattactant) (Wojnarowski et al., 1999).
2. IL-17A (the characteristic effector signature cytokine of Th17 cells) (Doe et al., 2010). The decision was made not to stain for IL-17F since this syntenic molecule acts on the same receptor as IL-17A but is a weaker inducer of cytokines (Iwakura et al., 2011).
3. IL-6, a pro-inflammatory cytokine which promotes the development of Th17 cells (Abe et al., 2001).
4. Neutrophil Elastase (NE, a cellular marker for neutrophils) to assess the degree of airways neutrophilia (Macedo et al., 2009).
5. Major Basic Protein (MBP, a cellular marker for eosinophils) to assess the degree of airways eosinophilia (Macedo et al., 2009).

Angiogenesis hypothesis

1. CD31 (a cellular marker of endothelial cells) to assess the degree of vascular remodelling (Corrigan et al., 2009).
2. VEGF, a major pro-angiogenic growth factor (Chetta et al., 2005).

A secondary objective of the studies described in this chapter was to understand the development of neutrophilia in the airways of smoking asthmatics, in particular whether eosinophils play a role in the development of the hypothesized Th17/IL-17

mediated neutrophilic inflammation in the airways of smoking asthmatics. We therefore conducted the following correlation analyses:

1. Eosinophils and pro-inflammatory cytokines.
2. Neutrophils and pro-inflammatory cytokines.
3. Eosinophils and Neutrophils.
4. Between the pro-inflammatory cytokines measured.

5.2 Characteristics of the populations studied

5.2.1 Demographics of healthy non-smoker controls, asthmatic non-smokers and asthmatic smokers

Endobronchial biopsies were obtained from 16 healthy non-smoker control subjects, 10 asthmatic non-smokers and 8 asthmatic smokers as described in section 2.6. The characteristics of these 3 groups is summarised in Table 8. All subjects classified as healthy had a PC_{20} histamine of greater than 16 mg/ml, while all those classified as asthmatics had a PC_{20} histamine of less than 8 mg/ml.

All the subjects who were classified as asthmatic smokers were current smokers with a mean pack year history of 9.4 years ± 10.9, and mean current number of cigarettes smoked of 14.6 cigarettes per day ± 8.9. Of those who were classified as healthy non-smokers and asthmatic non-smokers, there were 2 subjects in each group who were ex-smokers with no subjects who were current smokers. The two ex-smokers in the healthy non-smoker group had a pack year history of 0.2 and 2.25 years and had stopped smoking for 8 and 5 years respectively, with a mean pack year history of 0.2 years ± 0.6. The two ex-smokers in the asthmatic non-smokers group had a pack year history of 0.125 and 0.15 years and had stopped smoking for 14 and 3 years respectively, with a mean pack year history of 0.0 years ± 0.1.

It is important to note that, comparing the asthmatic smoking and non-smoking groups there was no difference in severity of asthma as defined by % predicted FEV_1 ($p = 0.9355$), degree of airflow obstruction as defined by FEV_1/FVC ratio ($p = 0.6856$), bronchial hyper-reactivity as measured by PC_{20} histamine ($p = 0.4543$) or bronchodilator reversibility response ($p = 0.1609$). All subjects in the asthmatic non-smoking and smoking groups were receiving only a short acting β2-agonist for the management of their asthma.

Characteristics	Healthy Non smoker	Asthma Non smoker	Asthma Smoker
No.	16	10	8
Age, yr	27 ± 5	27 ± 5	27 ± 12
Height, m	1.74 ± 0.11	1.74 ± 0.10	1.77 ± 0.16
Weight, kg	70.1 ± 14.1	73.2 ± 9.8	85.9 ± 19.5
Men/women	10/6	6/4	6/2
Never smoker / ex-smoker / current smoker	14/2/0	8/2/0	0/0/8
Pack-years	0.2 ± 0.6	0.0 ± 0.1	9.4 ± 10.9
Atopy, No. (%)	3/16 (19)	10/10 (100)	8/8 (100)
PC_{20} histamine, mg/ml	> 16	1.194 ± 1.061	1.721 ± 1.561
FEV_1, % predicted	104.5 ± 9.9	95.1 ± 18.6	94.8 ± 19.7
Pre-bronchodilator FEV_1/FVC, %	84.8 ± 5.6	75.7 ± 8.6†	77.1 ± 7.8†
Bronchodilator response, %	3.6 ± 1.5	9.6 ± 6.5†	14.0 ± 6.7†

Table 8: Characteristics of the populations studied

Subjects were recruited into the study and underwent a single fibreoptic bronchoscopy to obtain endobronchial biopsies. The subjects were characterised and classified into 3 groups, healthy non-smoking controls, asthmatic non-smokers and asthmatic smokers at the screening visit. The characteristics of the 3 groups are summarised in Table 1. † $p < 0.05$ compared with healthy non smoker. Mean ± SD.

5.2.2 St. George's Respiratory Questionnaire

The St. George's Respiratory Questionnaire (SGRQ) was employed at the bronchoscopy visit and used objectively to assess whether there was a difference in asthma symptomology and quality of life between asthmatic non-smokers and asthmatic smokers. We did not find a significant difference between asthma non-smokers and asthma smokers in the SGRQ total score ($p = 0.3127$) and SGRQ component scores assessing symptoms, activity and impacts ($p = 0.7145$, $p > 0.9999$, and $p = 0.1040$ respectively). Of note both asthmatic non-smokers and asthmatic smokers had a higher total score ($p < 0.0001$ and $p < 0.0001$ respectively compared to healthy non-smoking controls. The individual component scores of symptoms ($p = 0.0003$ and 0.0023 respectively), activity ($p = 0.0215$ and $p = 0.0109$ respectively) and impacts ($p = 0.0001$ and $p < 0.0001$ respectively) were also higher in the asthmatic non-smoker and smoker groups compared to healthy non-smoking controls (Table 9).

St. George's Respiratory Questionnaire Score	Healthy Non smoker n = 16	Asthma Non smoker n = 10	Asthma Smoker n =8
Symptom	4.23 ± 1.47	27.38 ± 6.82†	31.49 ± 8.43†
Activity	4.15 ± 1.07	15.47 ± 4.36†	16.86 ± 5.00†
Impact	0.12 ± 0.12	8.26 ± 4.27†	9.98 ± 2.39†
Total	2.12 ± 0.34	13.48 ± 3.96†	15.59 ± 2.59†

Table 9: St. George's Respiratory Questionnaire

The St. George's Respiratory Questionnaire (SGRQ) was employed prior to bronchoscopy at the bronchoscopy visit and used objectively to assess whether there was a difference in asthma symptomology and quality of life between asthmatic non-smokers and asthmatic smokers. † $p < 0.05$ compared with healthy non smoker. Mean ± SEM

5.3 Effects of cigarette smoking on angiogenesis in the airways

5.3.1 VEGF expression in the airways of smoking asthmatics

Endobronchial biopsy sections were stained for vascular endothelial growth factor (VEGF) using a monoclonal antibody to VEGF and fast red staining as described in section 2.7.2 (Figure 37). There was no difference in the expression of VEGF in the epithelial cell layer in healthy non-smokers, asthmatic non-smokers and asthmatic smokers (58.77 VEGF+ cells/mm ± 10.79, 49.82 VEGF+ cells/mm ± 7.376, 56.57 VEGF+ cells/mm ± 9.013 respectively, $p = 0.9050$). In the submucosa however the mean expression of VEGF in healthy non-smokers was 16.98 VEGF+ cells/mm^2 ± 3.779, with an increase in expression seen in asthmatic non-smokers (50.45 VEGF+ cells/mm^2 ± 10.70, $p = 0.0026$) and asthmatic smokers (82.12 VEGF+ cells/mm^2 ± 13.21, $p < 0.0001$). There was no difference in the expression of VEGF in the submucosa comparing asthmatic non-smokers to asthmatic smokers ($p = 0.1009$) (Figure 38).

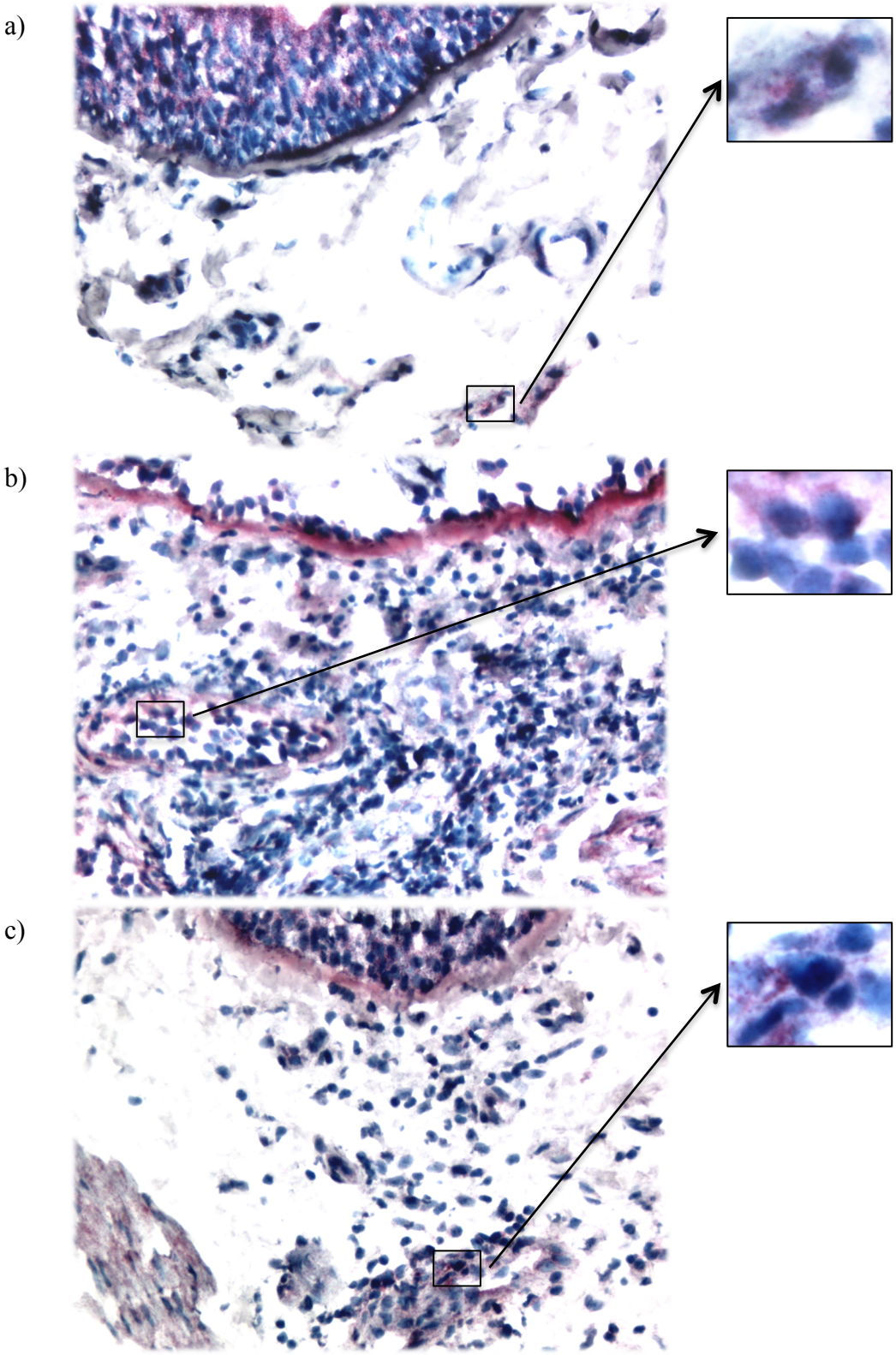

Figure 37: Vascular endothelial growth factor immunohistochemistry fast red staining

Endobronchial biopsy sections were stained for vascular endothelial growth factor (VEGF) using a monoclonal antibody to VEGF and fast red staining, as described in section 2.7.2. Cells that are positive for VEGF stained red in a) Healthy non-smoker b) Asthma non-smoker and c) Asthma smoker endobronchial biopsy sections.

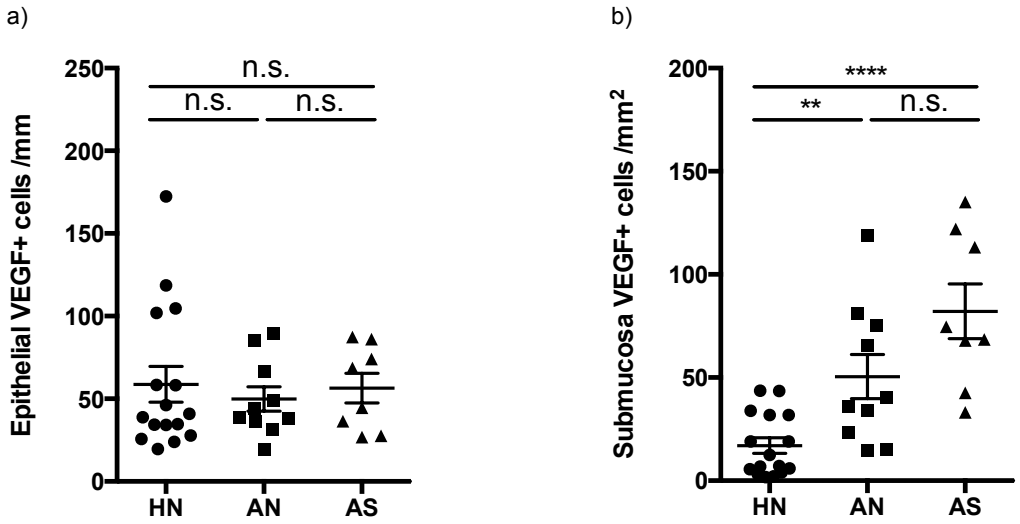

Figure 38: Vascular endothelial growth factor expression in the bronchial submucosa and epithelial layer

Sections of endobronchial biopsies were stained for Vascular Endothelial Growth Factor (VEGF) as described in section 2.7.2. a) There was no significant difference in VEGF expression between healthy non-smokers, asthma non-smokers and asthma smokers in the epithelial layer (Kruskal-Wallis $p = 0.9050$). b) There was an increase in VEGF expression in the submucosa of asthmatic non-smokers and asthmatic smokers compared to healthy non-smokers ($p = 0.0026$ and $p < 0.0001$ respectively). There was however no difference in the expression of VEGF between asthmatic non-smokers and asthmatic smokers ($p = 0.1009$). HN = Healthy Non-smoker, AN = Asthma Non-smoker and AS = Asthma Smoker. Mann-Whitney ** $p < 0.01$, **** $p < 0.0001$, n.s. = not significant, Mean ± SEM (shown for convenience).

5.3.2 CD31 expression in the airways of smoking asthmatics

To assess vascular remodelling, biopsy sections were stained for endothelial cells using the cellular marker CD31, which is expressed on the cell surface of these cells in particular at the intercellular junctions (Albelda et al., 1991, Corrigan et al., 2009). Endobronchial biopsy sections were stained for CD31 using a monoclonal antibody to CD31 and DAB staining as described in section 2.7.1 (Figure 39). The mean expression of CD31 in healthy non-smokers was 162.8 CD31+ cells/mm^2 ± 20.27, with an increase in expression seen in asthmatic non-smokers (298.4 CD31+ cells/mm^2 ± 35.64, $p = 0.0041$) and asthmatic smokers (284.5 CD31+ cells/mm^2 ± 22.27, $p = 0.0019$). There was no difference in the expression of CD31 between asthmatic non-smokers and asthmatic smokers ($p = 0.8718$).

To further quantify the extent of vascular remodelling, the mean number and size of vessels in the bronchial submucosa were also quantified. The mean number of vessels in the bronchial submucosa of healthy non-smokers was 62.45 vessels /mm^2 ± 6.716, with an increase seen in asthmatic non-smokers (157.1 vessels/mm^2 ± 16.41, $p < 0.0001$) and asthmatic smokers (131.5 vessels/mm^2 ± 5.838, $p < 0.0001$). There was no difference in the expression of CD31 between asthmatic non-smokers and asthmatic smokers ($p = 0.3759$). There was no difference in the mean vessel size seen in healthy non-smokers, asthmatic non-smokers and asthmatic smokers (7.571 pixels ± 0.3093, 7.273 pixels ± 0.4888, 7.204 pixels ± 0.4324 respectively, $p = 0.7096$) (Figure 40).

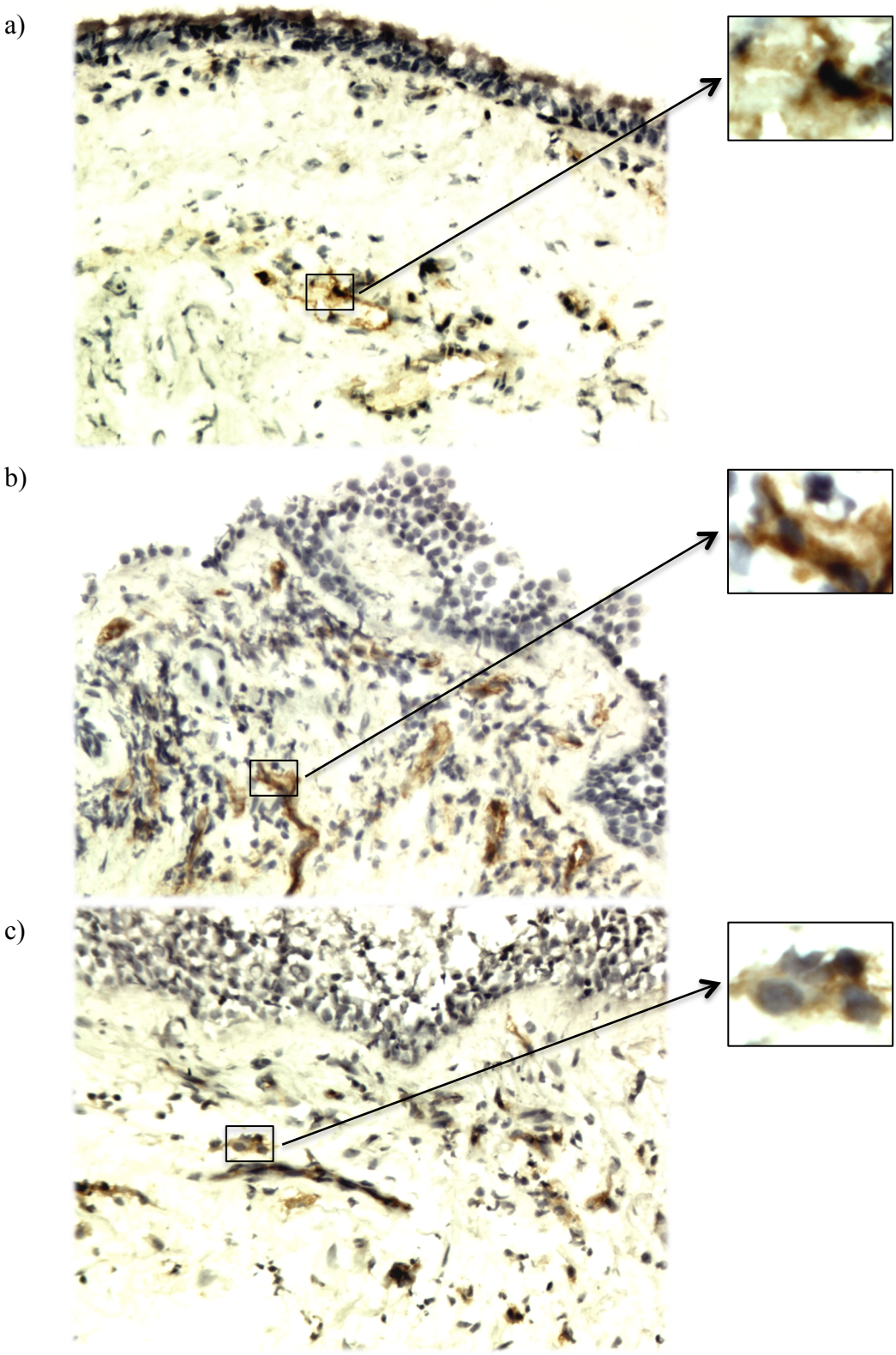

Figure 39: CD31 immunohistochemistry DAB staining

Endobronchial biopsy sections were stained for CD31 using a monoclonal antibody to CD31 and DAB staining as described in section 2.7.1. CD31 is being used as a marker for endothelial cells. Cells that are positive for CD31 stained brown in a) Healthy non-smoker b) Asthma non-smoker and c) Asthma smoker endobronchial biopsy sections.

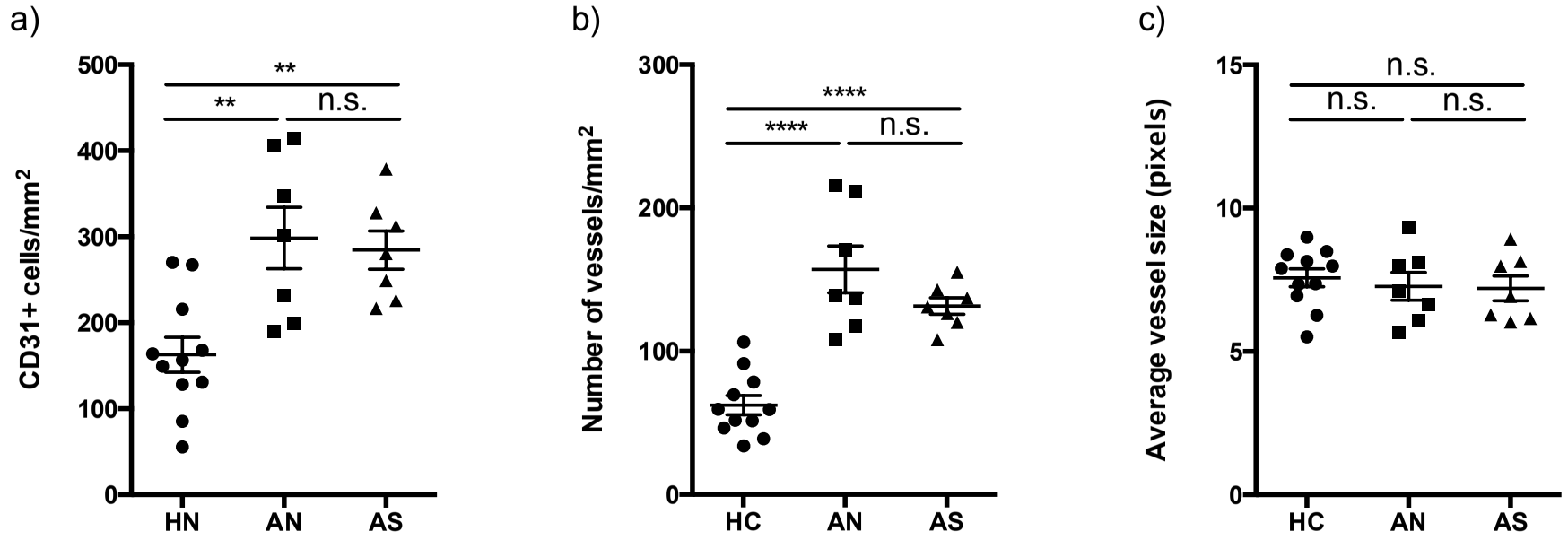

Figure 40: CD31 expression in endobronchial biopsies

Sections of endobronchial biopsies were stained for CD31 as described in section 2.7.1. a) There was increased expression of submucosal CD31 in asthmatic non-smokers and asthmatic smokers compared to healthy non-smokers ($p = 0.0041$ and $p = 0.0019$ respectively). b) There was also an increase in the number of blood vessels in the submucosa in asthmatic non-smokers and asthmatic smokers compared to healthy non-smokers ($p < 0.0001$ and $p < 0.0001$ respectively). c) There was however no difference in the average vessel size between healthy non-smokers, asthma non-smokers and asthma smokers (Kruskal-Wallis $p = 0.7096$). HN = Healthy Non-smoker, AN = Asthma Non-smoker and AS = Asthma Smoker. Mann-Whitney ** $p < 0.01$, **** $p < 0.0001$, n.s. = not significant, Mean ± SEM (shown for convenience).

5.4 Effects of cigarette smoking on pro-inflammatory cytokine expression

5.4.1 IL-6 expression in the airways of smoking asthmatics

Endobronchial biopsy sections were stained for interleukin-6 (IL-6) using a monoclonal antibody to IL-6 and fast red staining as described in section 2.7.2 (Figure 41). There was a significant increase in the mean number of cells which stained positive for IL-6 in the submucosa of endobronchial sections of asthmatic smokers compared to asthmatic non-smokers and healthy non-smokers (asthmatic smoker 73.27 IL-6+ cells/mm^2 ± 15.11; vs asthmatic non-smokers 30.99 IL-6+ cells/mm^2 ± 10.22, p = 0.0343; vs healthy non-smokers 21.98 IL-6+ cells/mm^2 ± 3.554, p = 0.0007). There was no difference in the mean numbers of cells expressing IL-6 between asthmatic non-smokers and healthy non-smokers (p = 0.6884) (Figure 44 a).

5.4.2 IL-8 expression in the airways of smoking asthmatics

Endobronchial biopsy sections were stained for interleukin-8 (IL-8) using a monoclonal antibody to IL-8 and fast red staining as described in section 2.7.2 (Figure 42). There was a significant increase in the mean number of cells which stained positive for IL-8 in the submucosa of endo-bronchial sections of asthmatic smokers compared to asthmatic non-smokers and healthy non-smokers (asthmatic smoker 15.18 IL-8+ cells/mm^2 ± 4.286; vs asthmatic non-smokers 1.932 IL-8+ cells/mm^2 ± 1.391, p = 0.0008; vs healthy non-smokers 2.255 IL-8+ cells/mm^2 ± 0.9994, p = 0.0003). There was no difference in the mean numbers of cells expressing IL-8 between asthmatic non-smokers and healthy non-smokers (p = 0.7357) (Figure 44 b).

5.4.3 IL-17A expression in the airways of smoking asthmatics

Endobronchial biopsy sections were stained for interleukin-17A (IL-17A) using a monoclonal antibody to IL-17A and fast red staining as described in section 2.7.2 (Figure 43). There was a significant increase in the mean number of cells which stained positive for IL-17A in the submucosa of endobronchial sections of asthmatic smokers compared to asthmatic non-smokers and healthy non-smokers (asthmatic smoker 8.248 IL-17A+ cells/mm2 ± 2.634; vs asthmatic non-smokers 2.536 IL-17A+ cells/mm2 ± 1.245, $p = 0.0400$; vs healthy non-smokers 0.7830 IL-17A+ cells/mm2 ± 0.3525, $p = 0.0009$). There was no difference in the mean numbers of cells expressing IL-17A between asthmatic non-smokers and healthy non-smokers ($p = 0.2288$) (Figure 44 c).

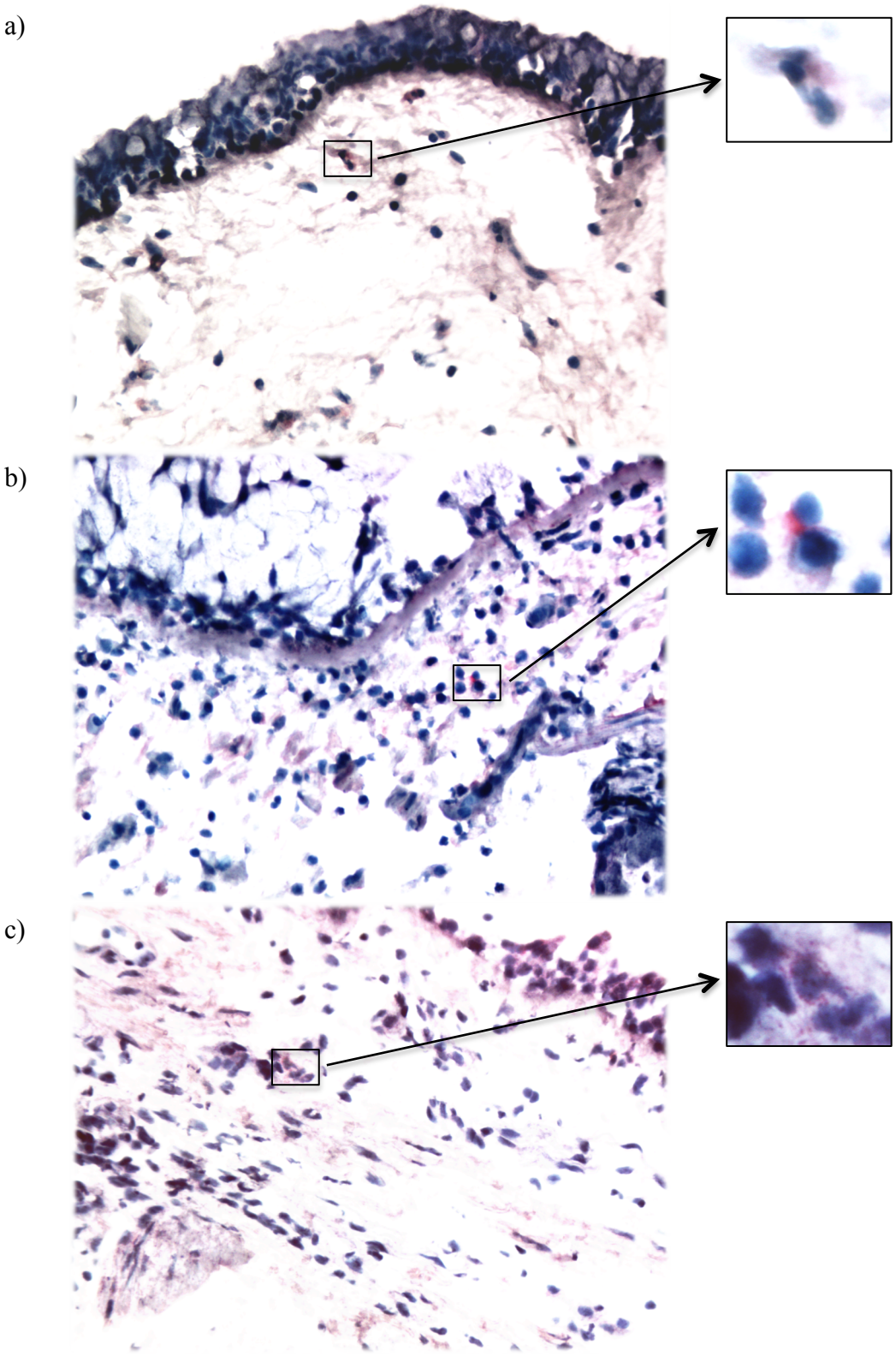

Figure 41: Interleukin-6 immunohistochemistry fast red staining

Endobronchial biopsy sections were stained for interleukin-6 (IL-6) using a monoclonal antibody to IL-6 and fast red staining as described in section 2.7.2. Cells that are positive for IL-6 stained red in a) Healthy non-smoker b) Asthma non-smoker and c) Asthma smoker endobronchial biopsy sections.

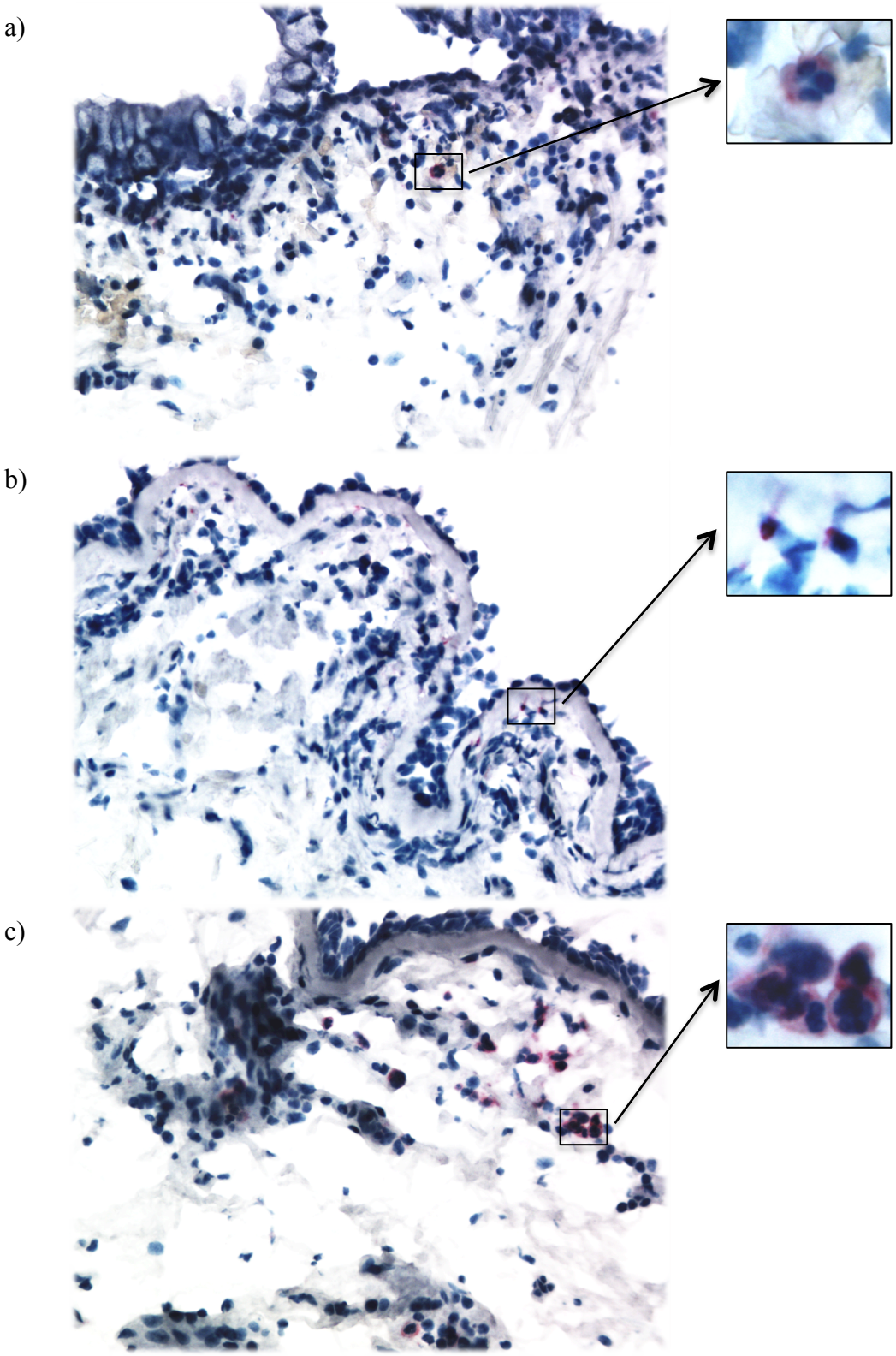

Figure 42: Interleukin-8 immunohistochemistry fast red staining

Endobronchial biopsy sections were stained for interleukin-8 (IL-8) using a monoclonal antibody to IL-8 and fast red staining as described in section 2.7.2. Cells that are positive for IL-8 stained red in a) Healthy non-smoker b) Asthma non-smoker and c) Asthma smoker endobronchial biopsy sections.

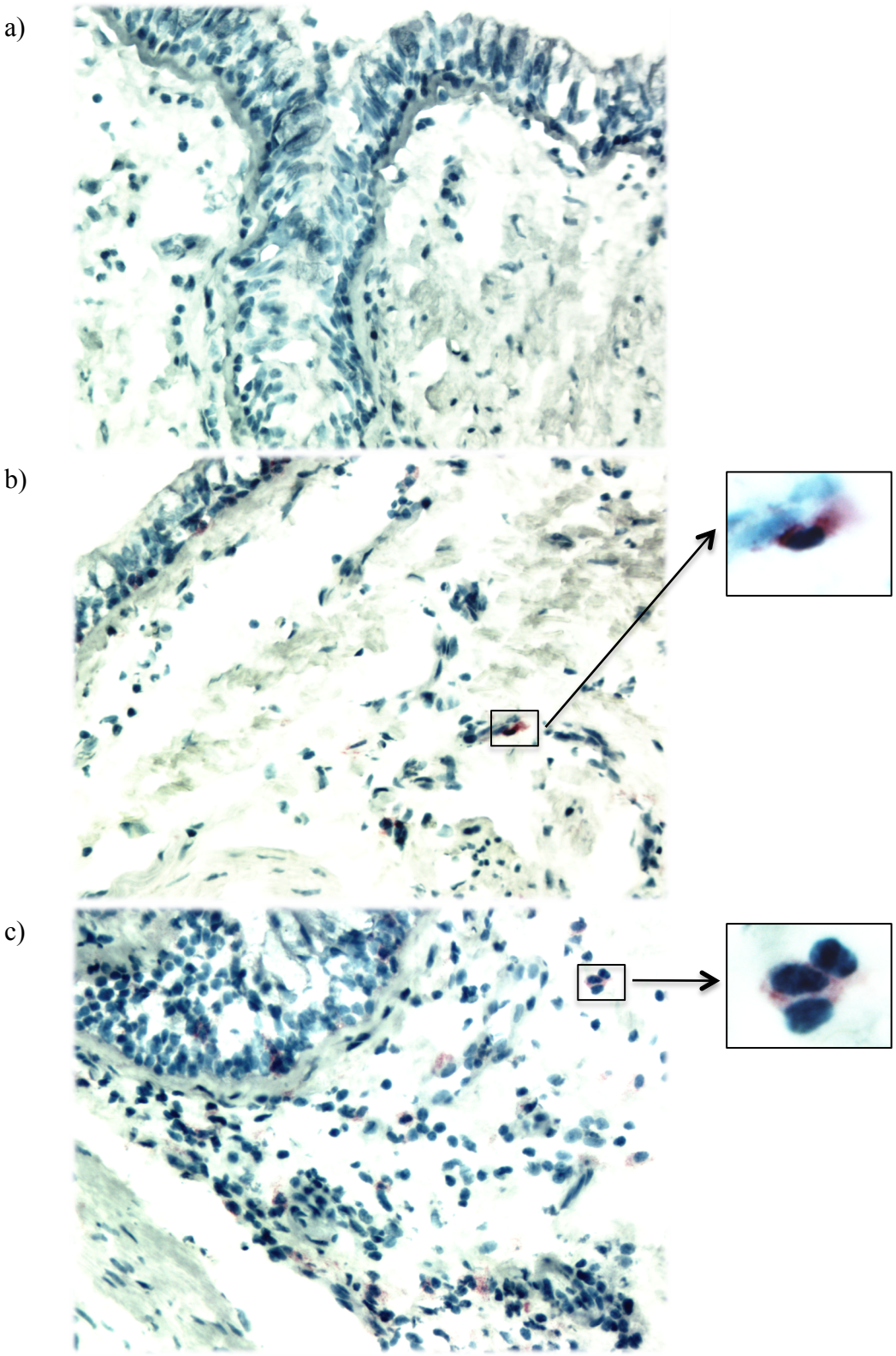

Figure 43: Interleukin-17A immunohistochemistry fast red staining

Endobronchial biopsy sections were stained for interleukin-17A (IL-17A) using a monoclonal antibody to IL-17A and fast red staining as described in section 2.7.2. Cells that are positive for IL-17A stained red in a) Healthy non-smoker b) Asthma non-smoker and c) Asthma smoker endobronchial biopsy sections.

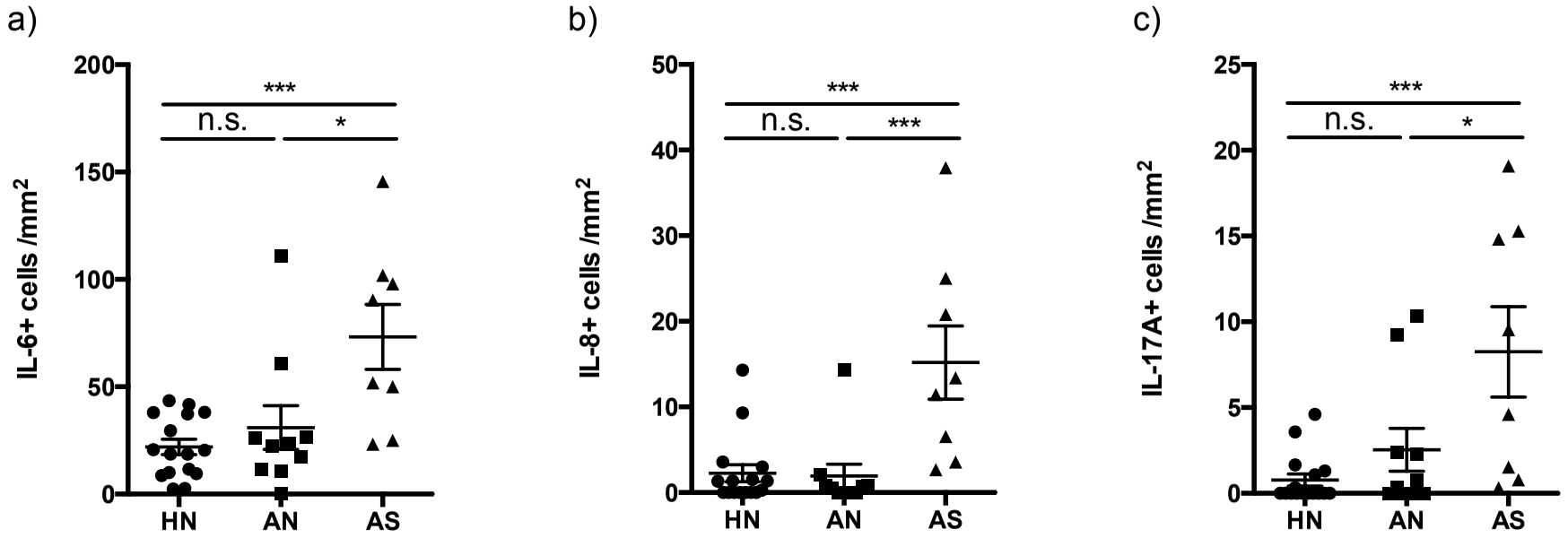

Figure 44: Pro-inflammatory cytokine expression in endobronchial biopsies

Sections of endobronchial biopsies were stained for the pro-inflammatory cytokines IL-6, IL-8 and IL-17A as described in section 2.7.2. a) There was increased expression of IL-6 in the submucosa of asthmatic smokers compared to asthmatic non-smokers and healthy non-smokers ($p = 0.0343$ and $p = 0.0007$ respectively). b) There was also increased expression of IL-8 in the submucosa of asthmatic smokers compared to asthmatic non-smokers and healthy non-smokers ($p = 0.0008$ and $p = 0.0003$ respectively). c) Furthermore an increased expression of IL-17A in the submucosa of asthmatic smokers compared to asthmatic non-smokers and healthy non-smokers was also observed ($p = 0.0400$ and $p = 0.0009$ respectively). HN = Healthy Non-smoker, AN = Asthma Non-smoker and AS = Asthma Smoker. Mann-Whitney * $p < 0.05$, *** $p < 0.001$, n.s. = not significant, Mean ± SEM (shown for convenience)

5.5 Cellular inflammatory profile in the airways of smoking asthmatics

5.5.1 Eosinophils in the airways of smoking asthmatics

Endobronchial biopsy sections were stained for eosinophils using a monoclonal antibody to eosinophil major basic protein (MBP) and fast red staining as described in section 2.7.2 (Figure 45). There was a significant increase in the mean number of eosinophils in the submucosa of the airways of asthmatic smokers and asthmatic non-smokers compared to healthy non-smokers (healthy non-smokers 5.259 MBP+ cells/mm^2 ± 1.903; vs asthmatic non-smoker 59.26 MBP+ cells/mm^2 ± 17.99, $p = 0.0002$; asthmatic smoker 107.8 MBP+ cells/mm^2 ± 19.22, $p < 0.0001$). There was however no difference in the degree of eosinophilia between asthmatic smokers and asthmatic non-smokers ($p = 0.0675$) (Figure 47 a).

5.5.2 Neutrophils in the airways of smoking asthmatics

Endobronchial biopsy sections were stained for neutrophils using a monoclonal antibody to neutrophil elastase (NE) and fast red staining as described in section 2.7.2 (Figure 46). There was a significant increase in the mean number of neutrophils in the submucosa of the airways of asthmatic smokers compared to asthmatic non-smokers (asthmatic non-smoker 14.17 NE+ cells/mm^2 ± 2.577 vs asthmatic smoker 37.31 NE+ cells/mm^2 ± 8.676, $p = 0.0194$). There was however no difference in the degree of neutrophilia detected between asthmatic smokers and healthy non-smokers (healthy non-smoker 22.61 NE+ cells/mm^2 ± 3.734, $p = 0.1166$). Of note there was also no difference in the degree of neutrophilia between asthmatic non-smokers and healthy non-smokers ($p = 0.0842$) (Figure 47 b).

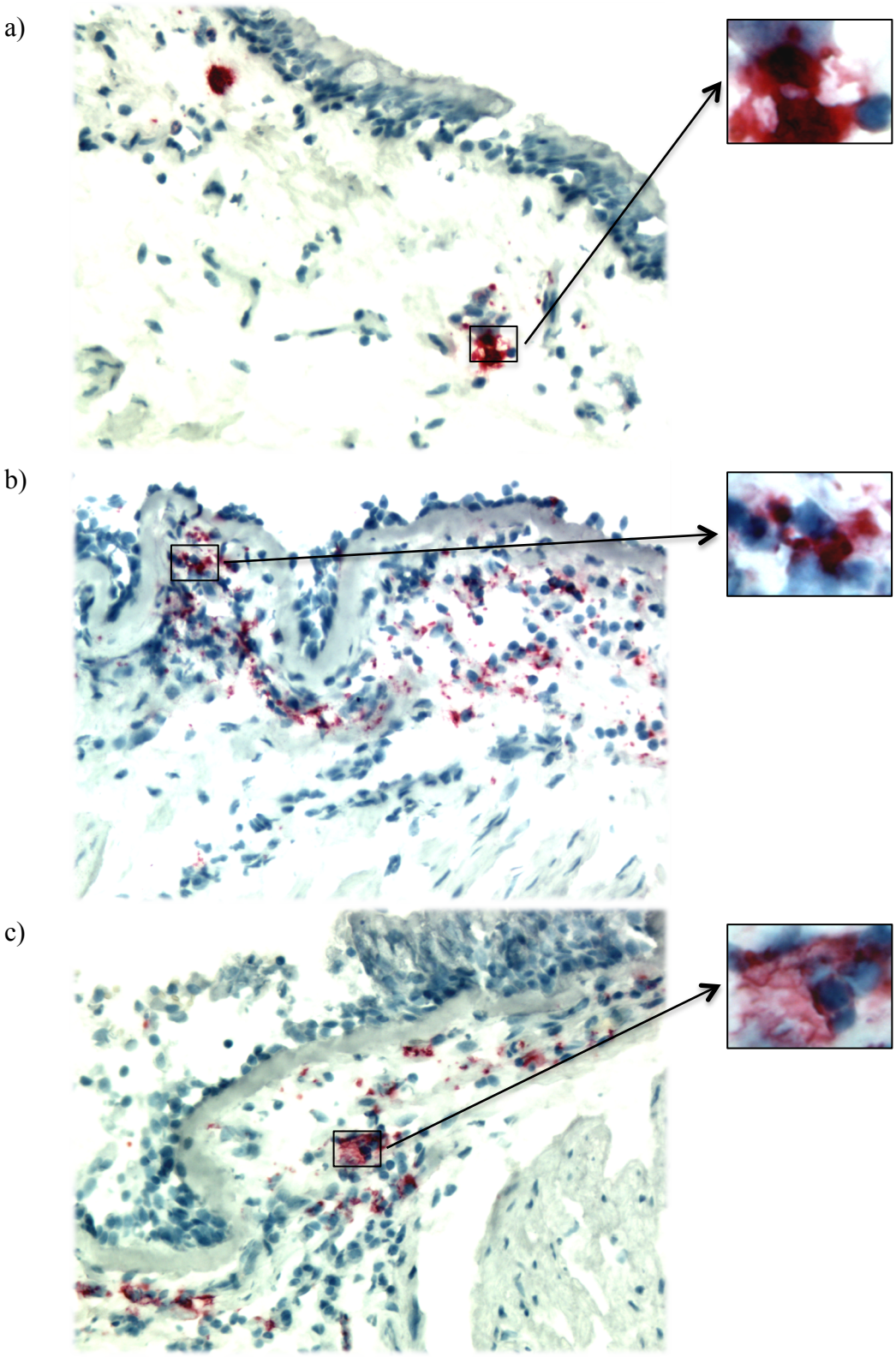

Figure 45: Eosinophil major basic protein immunohistochemistry fast red staining

Endobronchial biopsy sections were stained for eosinophils using a monoclonal antibody to eosinophil major basic protein (MBP) and fast red staining as described in section 2.7.2. Cells that are positive for MBP stained red in a) Healthy non-smoker b) Asthma non-smoker and c) Asthma smoker endobronchial biopsy sections.

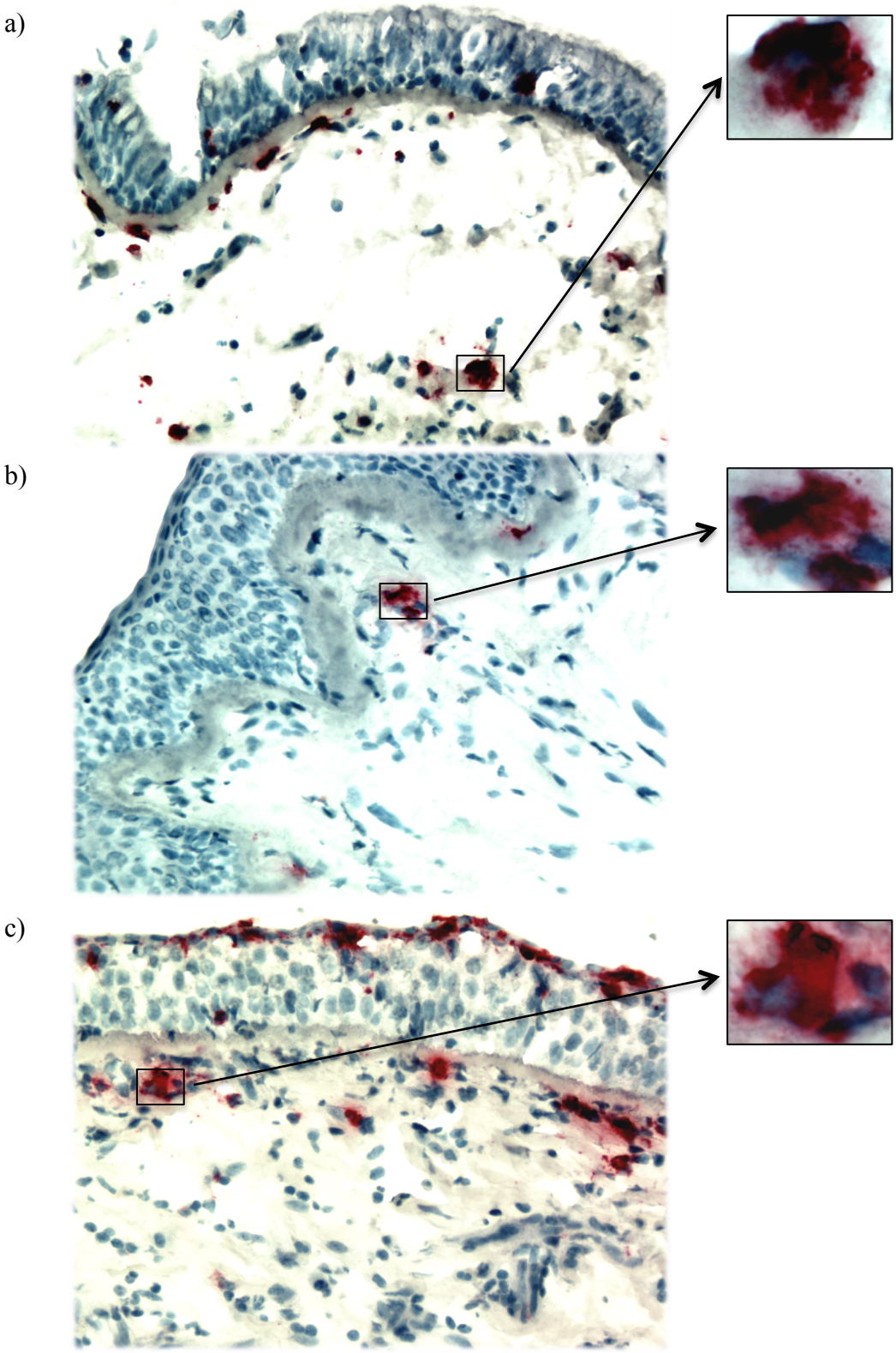

Figure 46: Neutrophil elastase immunohistochemistry fast red staining

Endobronchial biopsy sections were stained for neutrophils using a monoclonal antibody to neutrophil elastase and fast red staining as described in section 2.7.2. Cells that are positive for neutrophil elastase stained red in a) Healthy non-smoker b) Asthma non-smoker and c) Asthma smoker endo-bronchial biopsy sections.

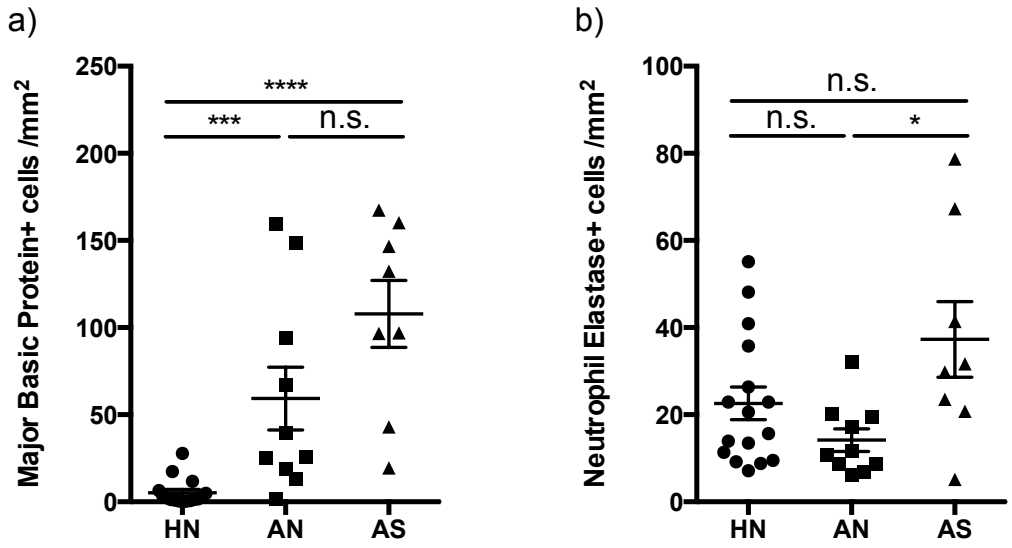

Figure 47: Eosinophils and Neutrophils in endobronchial biopsies

Sections of endobronchial biopsies were stained for eosinophils and neutrophils as described in section 2.7.2. a) There was a significant increase in the mean number of eosinophils in the airways of both asthmatic smokers and asthmatic non-smokers compared to healthy non-smokers ($p < 0.0001$ and $p = 0.0002$ respectively). No difference in the degree of eosinophilia was found between asthmatic smokers and asthmatic non-smokers ($p = 0.0675$). b) There was a significant increase in the mean number of neutrophils in the airways of asthmatic smokers compared to asthmatic non-smokers ($p = 0.0194$). There was however no difference in the degree of neutrophilia detected between asthmatic smokers and healthy non-smokers ($p = 0.1166$). HN = Healthy Non-smoker, AN = Asthma Non-smoker and AS = Asthma Smoker. Mann-Whitney * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$, n.s. = not significant, Mean ± SEM (shown for convenience)

5.6 Correlations between pro-inflammatory cytokine expression and cellular inflammatory profile

5.6.1 Eosinophils and pro-inflammatory cytokines

In endobronchial biopsies from asthmatic subjects (both non-smokers and smokers) there was a strong correlation between the numbers of eosinophils and the numbers of IL-8+ cells and IL-17A+ cells ($r = 0.7150$, $p = 0.0009$ and $r = 0.6953$, $p = 0.0014$ respectively), with the linear regression model accounting for 51% and 48% respectively of the variance in the data ($r^2 = 0.5112$ and $r^2 = 0.4834$ respectively) (Figure 48 b and c). The numbers of eosinophils did not correlate with the numbers of IL-6+ cells in asthmatic subjects ($r = 0.3295$, $p = 0.1818$) (Figure 48 a).

5.6.2 Neutrophils and pro-inflammatory cytokines

In endobronchial biopsies from asthmatic subjects (both non-smokers and smokers) there was a weak correlation between the numbers of neutrophils and the numbers of IL-6+ cells ($r = 0.4877$, $p = 0.0401$) (Figure 49 a) and a strong correlation between the numbers of neutrophils and IL-8+ and IL-17A+ cells ($r = 0.7976$, $p < 0.0001$ and $r = 0.9069$, $p < 0.0001$ respectively) (Figure 49 b and c). The linear regression model accounted for 24%, 64% and 82% of the variance in the data comparing neutrophils with IL-6+, IL-8+ and IL-17A+ cells respectively (neutrophils vs IL-6+ $r^2 = 0.2379$; neutrophils vs IL-8+ $r^2 = 0.6362$; neutrophils vs IL-17A+ $r^2 = 0.8225$) (Figure 49 a, b and c).

5.6.3 Eosinophils and neutrophils in the airways of asthmatics

In endobronchial biopsies from asthmatic subjects (both non-smokers and smokers) there was a weak correlation between the numbers of eosinophils and the numbers of neutrophils ($r = 0.5721$, $p = 0.0131$), with the linear regression model accounting for 33% of the variance in the data ($r^2 = 0.3272$) (Figure 50).

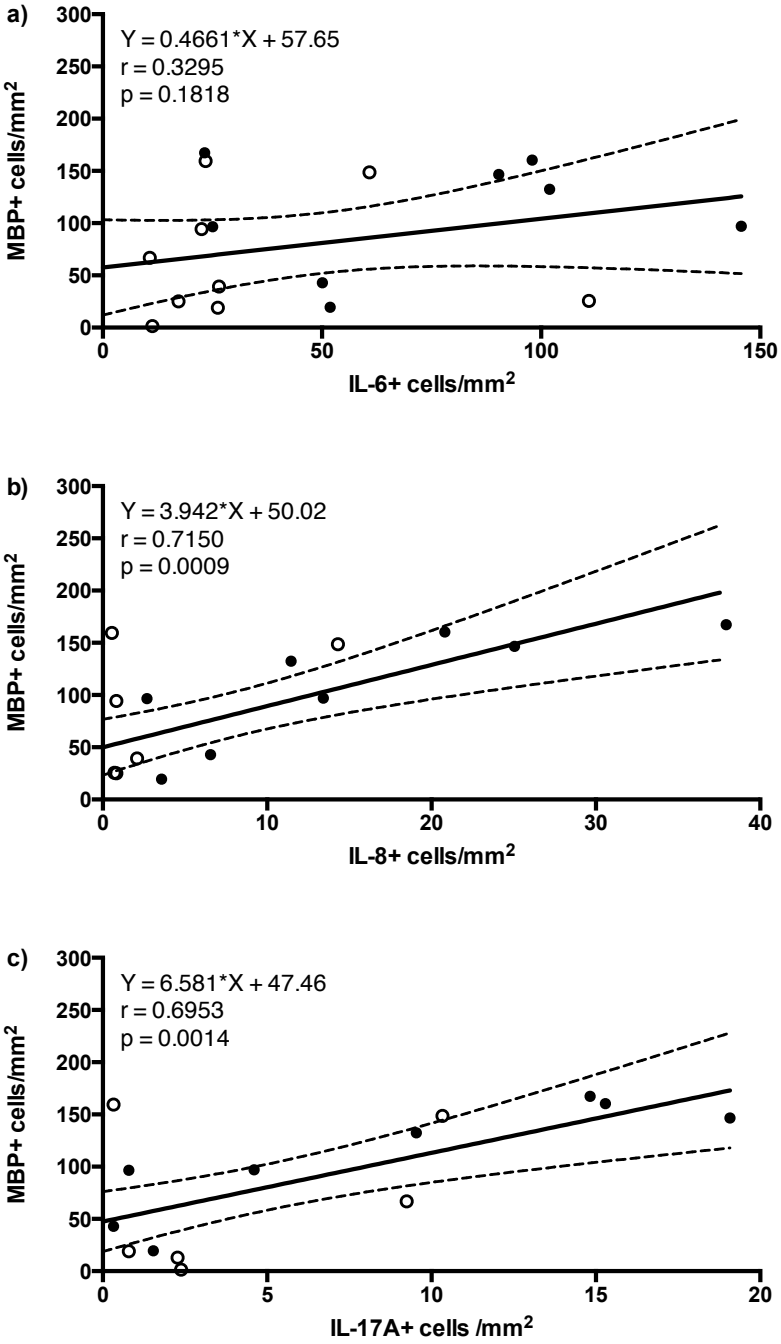

Figure 48: Correlations between eosinophils and pro-inflammatory cytokines in asthmatic subjects

a) There was no correlation found between the numbers of IL-6+ cells and eosinophils in endobronchial sections from asthmatic subjects. b) & c) The numbers of eosinophils correlated with the numbers of IL-8+ cells and the numbers of IL-17A+ cells. ○ asthmatic non-smoker, ● asthmatic smoker; MBP = Eosinophil Major Basic Protein; Pearson correlation, Linear regression and 95% confidence band.

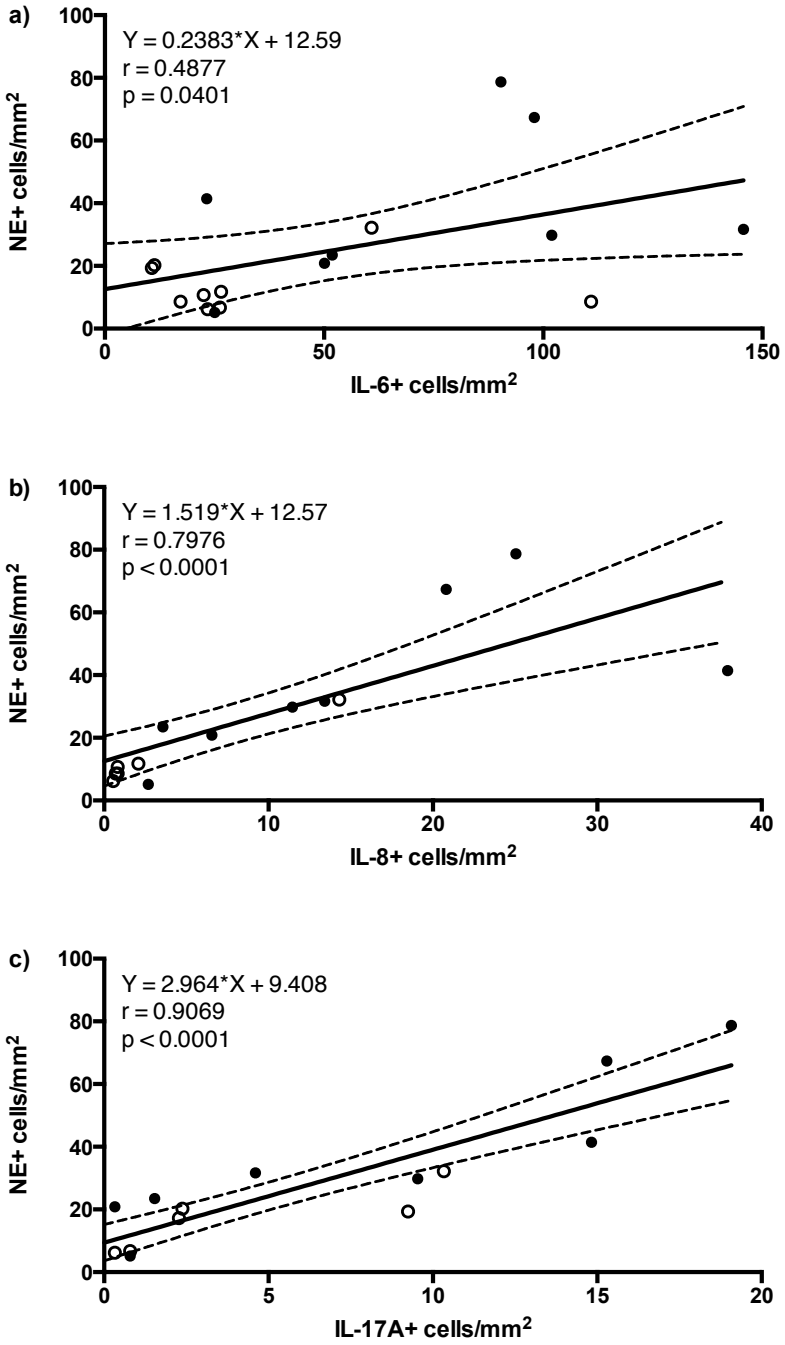

Figure 49: Correlations between neutrophils and pro-inflammatory cytokines in asthmatic subjects

a), b) & c) The numbers of neutrophils correlated with the numbers of IL-6+ cells, IL-8+ cells and IL-17A+ cells in endobronchial sections from the asthmatic subjects. ○ asthmatic non-smoker, ● asthmatic smoker; NE = Neutrophil Elastase; Pearson correlation, Linear regression and 95% confidence band.

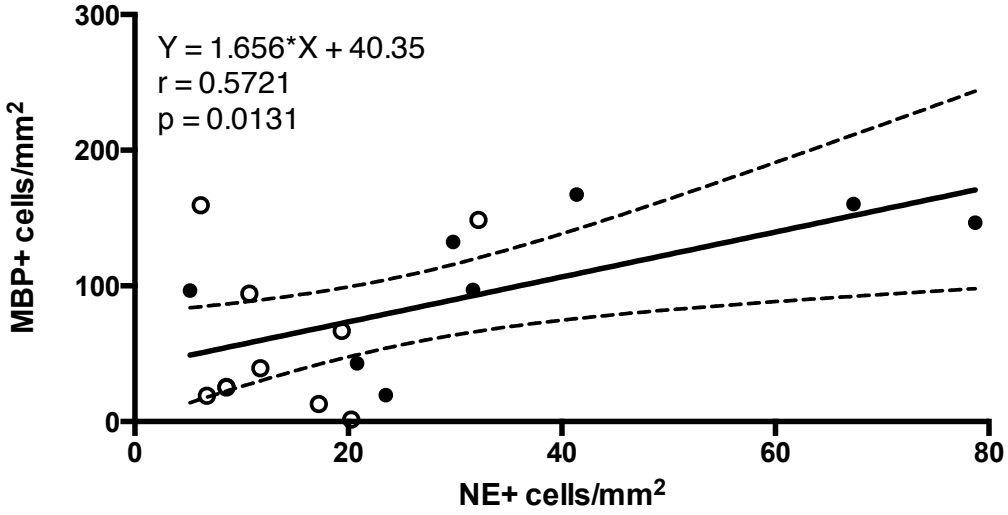

Figure 50: Correlations between eosinophils and neutrophils in asthmatic subjects

The numbers of eosinophils correlated with the numbers of neutrophils in endobronchial sections from asthmatic subjects (both non-smokers and smokers). ○ asthmatic non-smoker, ● asthmatic smoker; MBP = Eosinophil Major Basic Protein, NE = Neutrophil Elastase; Pearson correlation, Linear regression and 95% confidence band.

5.6.4 Pro-inflammatory cytokine expression in the airways of asthmatics

In endobronchial biopsies from asthmatic subjects (both non-smokers and smokers) there was a strong correlation between the numbers of IL-8+ cells and the numbers of IL-17A+ cells ($r = 0.8418$, $p < 0.0001$), with the linear regression model accounting for 71% of the variance in the data ($r^2 = 0.7086$) (Figure 51).

There was no correlation between the numbers of IL-6+ cells and IL-17A+ cells in asthmatic subjects (both non-smokers and smokers) ($r = 0.3466$, $p = 0.1589$) (data not shown), or between the numbers of IL-6+ cells and IL-8+ cells in asthmatic subjects (both non-smokers and smokers) ($r = 0.3789$, $p = 0.1210$) (data not shown).

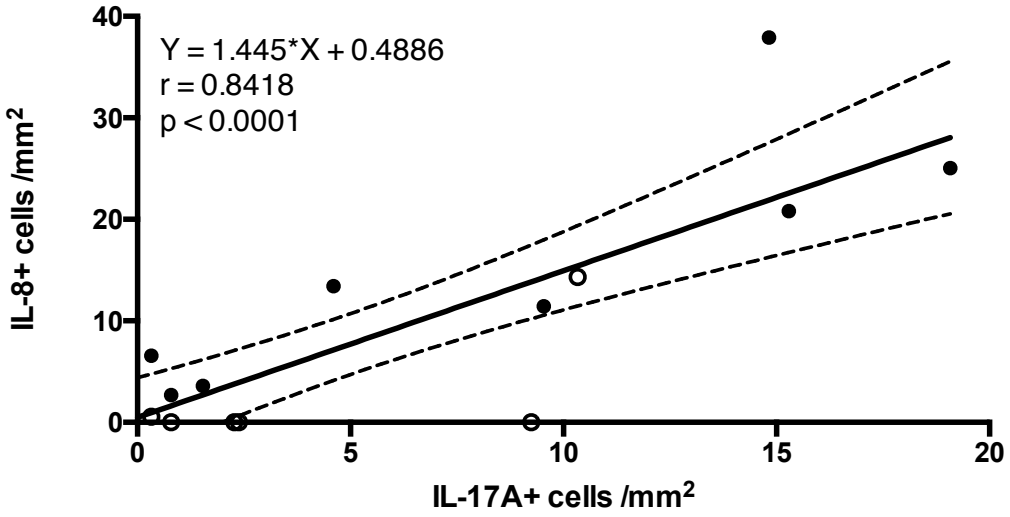

Figure 51: Correlations between pro-inflammatory cytokines IL-8 and IL-17A in asthmatic subjects

The numbers of IL-8+ cells correlated strongly with the numbers of IL-17A+ cells in endobronchial sections from asthmatic subjects (both non-smokers and smokers). ○ asthmatic non-smoker, ● asthmatic smoker; Pearson correlation, Linear regression and 95% confidence band.

5.7 Discussion and Summary

The characteristics of the 3 studied groups were similar with regard to age, male/female ratio, height and weight. The subjects with asthma in both the non-smoking group and smoking group were all steroid naïve patients with mild disease, and there was no difference in baseline lung function, degree of airflow obstruction, bronchial hyperreactivity or β2-agonist reversibility between each group. Although cigarette smoking has been suggested to increase asthma symptoms (Althuis et al., 1999), we did not find a difference in the SGRQ scores between non-smoking asthmatics and smoking asthmatics. This most likely reflects the small numbers of subjects, the fact that they were deliberately chosen to have mild disease, and perhaps the fact that the SGRQ is a respiratory quality of life questionnaire developed for patients with chronic airflow obstruction (both chronic obstructive pulmonary disease (COPD) and asthma) (Jones et al., 1991) rather than a specific asthma quality of life questionnaire like the Asthma Control Questionnaire (Juniper et al., 1999b) or the Asthma Quality of Life Questionnaire (Juniper et al., 1999a). We chose to use the SGRQ as the instrument to measure quality of life as it also captures symptoms and quality of life issues related to COPD, a disease that is caused primarily by cigarette smoking. In retrospect the SGRQ may not have been the most appropriate tool.

The main aim of the study was to look at the effects of cigarette smoking on airways inflammation in asthma, and particularly the release of remodelling and pro-inflammatory cytokines. In view of this, a smoker was defined as someone who currently smokes ≥ 5 cigarrettes a week, and a non-smoker as someone who had not smoked for at least 12 months prior to screening and with a < 0.5 pack year history. A minimum pack year exposure criterion for selection into the smoking group was not set as this would have resulted in recruitment of subjects either starting to smoke at a very young age or having smoked for a long period of time, making them vulnerable to COPD. On the other hand, we were also mindful of balancing the likely acute effects of smoking on airways inflammation and its likely more long term efffects on airways remodelling. Of course there is little precedent in the literature for judging the time course of such changes. To eliminate the possible confounding factor of concomitant medications, all of the asthmatic subjects recruited into the study were

taking only short acting β2 agnonist for the maintenance treatment of their asthma, and none were taking inhaled corticosteroid therapy, antihistamines or leukotriene antagonists. While this obviated possible confounding effects of therapy, it likely diluted the power of the study to detect inflammatory changes and particularly changes induced by smoking. Again, there is no clear "answer" to the problem of allowing for effects of therapy when performing such studies.

St-Laurent et al. in 2008 published their findings that smoking asthmatic patients had elevated numbers of submucosal neutrophil elastase positive cells, with a corresponding increase expression of IL-8 in the epithelial layer, but no difference in expression of IL-8 in the submucosa. They also reported elevated expression of interferon-γ in the bronchial submucosa and increased squamous metaplasia in asthmatic smokers. They did not find any difference in the numbers of mucosal CD3 positive cells, CD68 positive cells, MBP positive cells or tryptase positive cells between asthmatic smokers and non-smokers. Furthermore there was no difference in the expression of IL-4, IL-5, TNF and TGF-β between asthmatic non-smokers and smokers. They also did not find a difference in airways remodelling between both groups as measured by airway smooth muscle area, goblet cell hyperplasia and reticular basement membrane thickening (St-Laurent et al., 2008). A study by Broekema et al. in 2009 suggested in contrast that there was evidence of increased airways epithelial remodelling in asthmatic smokers as shown by increased numbers of goblet cells, increased mucus-positive epithelium and increased thickness of the epithelial layer. Contrary to St-Laurent et al. they found an increase in the numbers of tryptase positive cells and a reduction in the numbers of eosinophil peroxidase positive cells, with no differences in the numbers of neutrophil elastase positive cells and the percentage of normal epithelium and metaplasia between asthmatic smokers and non-smokers. They corroborated St-Laurent's findings with regard to CD3 positive cells, CD68 positive cells and reticular basement membrane thickness (Broekema et al., 2009).

The present findings show an increased number of neutrophil elastase positive cells and no difference in the number of MBP positive cells in asthmatic smokers compared to non-smokers, which corroborate the findings of St-Laurent et al. but are

contrary to those of Broekema et al. (St-Laurent et al., 2008, Broekema et al., 2009). We also found increased IL-8 expression in the entire endobronchial biopsy sections, while St-Laurent et al. did not find any difference in IL-8 expression in the submucosa with an increase seen in the epithelial layer. Of note when measuring IL-8 expression in the epithelial layer, St-Laurent et al. employed a qualitative scoring technique (scored from 0 to 8, representing percentage positivity in the epithelium) rather than a quantitative scoring technique that was used to assess the expression in the submucosa (positive cells/mm^2). The present findings extend those of both St-Laurent et al. and Broekema et al. by showing an increase in the mean numbers of IL-6 positive cells and IL-17A positive cells in the bronchial mucosa of asthmatic smokers compared to asthmatic non-smokers. Furthermore it is also demonstrated that there was no significant difference in the degree of vascular remodelling and VEGF expression in the airways of asthmatic smokers compared to asthmatic non-smokers. We believe that this is the first demonstration of an increase in IL-6 and IL-17A expression, with no change in vascular remodelling and VEGF expression, in the bronchial mucosa of asthmatic smokers compared to asthmatic non-smokers.

Spears et al. in 2013 reported that induced sputum of asthmatic smokers contained significantly elevated mean concentrations of IL-6, IL-7 and IL-12 with a trend for an increase in IL-1RA, IL-8/CXCL8, IL-17 and MCP-1/CCL2 (Spears et al., 2013). Interestingly their population of asthmatic smokers did not demonstrate an increase in sputum neutrophils compared to non-smoking asthmatics. This is in contrast to previous publications that have demonstrated a sputum neutrophilia in asthmatic smokers (Chalmers et al., 2001, Boulet et al., 2006, Chaudhuri et al., 2006, Hillas et al., 2011, Hillas et al., 2013). Elevated IL-8 and TGF-β1 concentrations have also been reported in induced sputum of asthmatic smokers compared to asthmatic non-smokers (Chalmers et al., 2001, Hillas et al., 2013). These publications are supportive of our findings that there is increased expression of IL-6 and IL-17A in the bronchial mucosa of asthmatic smokers compared to asthmatic non-smokers.

IL-17A promotes the development of neutrophilia by inducing the production of the neutrophil chemoattractant IL-8 by a variety of airways structural cells which include bronchial epithelial cells, endothelial cells, bronchial fibroblasts and airways smooth

muscle cells (Laan et al., 1999, Molet et al., 2001, Vanaudenaerde et al., 2003). Furthermore, elevated expression of IL-17A has been reported in the bronchial mucosa of mild to moderate asthmatics, and this has been shown to correlate with the numbers of neutrophils in induced sputum but surprisingly not with the number of neutrophils in the bronchial mucosa (Doe et al., 2010). The present studies in contrast show that the numbers of neutrophils in the bronchial mucosa strongly correlate with the expression of both IL-8 and IL-17A in the bronchial mucosa of asthmatics, and that the expression of IL-8 also strongly correlates with that of IL-17A. In view of the fact that we found increased expression of IL-17A in asthmatic smokers compared to non-smokers, we postulate that the airways neutrophilia seen in smoking asthmatics is driven by Th17 mediated inflammation through increased expression of IL-17A and IL-8.

An increase in sputum neutrophils in asthmatic patients has been shown to be associated with systemic inflammation (defined by elevation of both CRP and IL-6 in peripheral blood) (Fu et al., 2013). Furthermore a weak correlation has been described between peripheral blood IL-6 and sputum neutrophilia (Wood et al., 2012). We have extended these findings by demonstrating that in the bronchial mucosa of asthmatics, airways neutrophilia correlates with the expression of the pro-inflammatory cytokine IL-6. Considering that IL-6 and TGF-β1 are key cytokines required for the differentiation of naïve T cells to Th17 cells (Ivanov et al., 2006), the correlation between IL-6 and neutrophils may represent indirect evidence to support the hypothesis that the neutrophilic inflammation seen in asthmatic smokers (and possibly also in moderate to severe asthma) is orchestrated by Th17/IL-17A inflammation.

IL-8 is primarily a chemoattractant for neutrophils and does not usually act as a chemoattractant for eosinophils. However in the event that eosinophils are “primed” either by IL-5 or a pre-existing eosinophilic inflammatory disease such as asthma or hyper eosinophilic syndrome, IL-8 is able to act as a chemoattractant for these “primed” eosinophils (Sehmi et al., 1993, Warringa et al., 1993, Schweizer et al., 1994). A correlation between sputum eosinophil count and sputum IL-8 concentrations has been shown in moderately severe asthmatic patients (Jatakanon et al., 1999). In line with these findings we have demonstrated that the numbers of

eosinophils in the bronchial mucosa correlate with the numbers of IL-8 positive cells in asthmatic patients.

We have also demonstrated that the numbers of eosinophils correlated with the expression of IL-17A in the bronchial mucosa in the airways of asthmatic patients. This is contrary to what was published by Doe et al. who showed a correlation between the numbers of eosinophils and the expression of IL-17F but not IL-17A (Doe et al., 2010). This is an intriguing finding in the light of more recent publications showing that inhaled allergen challenge increases the numbers of Th17 cells and IL-17A concentrations in peripheral blood (Bajoriuniene et al., 2012) and that mepolizumab (anti-IL5 antibody) treatment reduces segmental allergen challenge induced eosinophilia and IL-17A expression in bronchoalveolar lavage fluid (Esnault et al., 2012). Of note there is a considerable overlap of the function and expression of both IL-17A and IL-17F, as they are highly homologous with each other, bind to the same receptors and their predominant cellular source is Th17 cells (Iwakura et al., 2011, Miossec and Kolls, 2012).

A weak correlation between airways eosinophilia and neutrophilia was observed in our population of asthmatics. Our findings corroborate that of Frangova et al. who showed that in asthmatic patients, there was a significant correlation between eosinophil count with neutrophil count in the bronchoalveolar lavage fluid (Frangova et al., 1996).

In our population of healthy non-smokers there were a number of subjects who demonstrated unusually high numbers of neutrophils in the airways in comparison with the mean of the group. One might speculate that this may be the result of these individuals having a subclinical respiratory tract infection or are convalescent following a recent respiratory tract infection, despite the fact that, on direct questioning all subjects who underwent bronchoscopies reported themselves as free from respiratory tract infections in the 4 weeks prior to the procedure.

In summary, asthmatic smokers are characterised by a predominance of Th17/IL-17A mediated neutrophilic inflammation of the bronchial airways. Furthermore there is

circumstantial evidence from the present data and published literature to indicate that the Th17/IL-17A mediated neutrophilic inflammation could be initiated by allergic eosinophilic inflammation and subsequently perpetuated by cigarette smoking.

Chapter 6: General discussion

6 General discussion

6.1 Re-evaluation of hypothesis

6.1.1 Summary of results

The main finding from this body of work is that there is an increase in the expression of IL-17A in the bronchial mucosa of asthmatic smokers compared to non-smokers. Concurrent with this there is increased expression of IL6 and IL-8 as well as elevated numbers of neutrophils in the bronchial mucosa of asthmatic smokers compared to non-smokers. Correlations were apparent between the expression of IL-17A with both the expression of IL-8 and the numbers of neutrophils in the bronchial mucosa of the asthmatics. Interestingly, in the asthmatics the degree of eosinophilia in the bronchial mucosa correlated with the expression of IL-8, IL-17A and the degree of neutrophilia while the degree of neutrophilia correlated with the expression of IL-6, IL-8 and IL-17A. The study did not however reveal any difference in the extent of vascular remodelling or degree of eosinophilia in the airways of the mild asthmatic smokers compared to the non-smokers.

With regard to the effects of IL-17A on possible local cellular targets, we found that stimulation of HTEpC cells with both CSE and IL-17A increased their expression of IL-6 and IL-8 in a synergistic manner. The data further suggest that this synergistic interaction between CSE and IL-17A in HTEpC cells may be mediated at least party by reactive oxygen species (ROS); further investigation of this is warranted. Co-stimulation of HTEpC cells with CSE, IL-17A and non-proteolytic allergens (cat dander and timothy grass pollen) resulted in a synergistic increase in expression of IL-6 and IL-8. This is an intriguing finding and the mechanism behind this interaction also warrants further investigation. Stimulation of primary human fibroblast cells with both CSE and IL-17A also resulted in a synergistic increase in expression of IL-6 with a trend for a synergistic increase in the expression of VEGF.

Not withstanding our failure to observe effects of smoking on vascular remodelling in the asthmatic bronchial mucosa, we demonstrated that CSE is able to induce HTEpC cells to express VEGF in a concentration dependent manner. This induction was dependent on the 3 main MAPK signalling molecules (ie p38 MAPK, Erk and JNK)

and, upstream from this, the PI3 kinase-dependent Akt phosphorylation pathway. In primary bronchial fibroblasts we also demonstrated that CSE is able to induce the expression of IL-6, TGF-β1 and VEGF in a concentration dependent manner. The induction of IL-6 and VEGF by CSE was found to be dependent on the p38 MAPK and ERK signalling pathways, while the induction of TGF-β1 was found to be dependent on the ERK and JNK signalling pathways. Of note, dexamethasone was able to inhibit the induction of IL-6, TGF-β1 and VEGF in primary bronchial fibroblasts by CSE. In summary, CSE promotes the release of pro-inflammatory cytokines that are supportive of the differentiation of T cells towards a Th17 phenotype. This along with our finding of elevated expression of IL-17A in the bronchial mucosa of smoking asthmatics provides circumstantial evidence supporting the hypothesis that CSE promotes the development of Th17/IL-17A mediated neutrophilic inflammation in the bronchial mucosa.

When primary bronchial fibroblasts were co-stimulated with either Poly I:C or LTA and CSE, there was a synergistic increase in the expression of VEGF. We also found that there was an additive increase in the expression of IL-6 induced by co-stimulation with LTA and CSE. However there was no clear similar effect on the expression of IL-6 induced by co-stimulation with Poly I:C and CSE.

Interestingly we showed that when HTEpC cells were stimulated with Poly I:C the expression of TSLP was inhibited by CSE, while there was no effect on the expression of IL-6. This implies that CSE, while promoting Th17 differentiation, may also preferentially inhibit differentiation of T cells towards the Th2 phenotype and this hypothesis warrants further investigation.

6.1.2 Review of hypothesis

The main findings of this body of work support a scenario where IL-17A plays a key, perhaps central role in the airways inflammation observed in asthmatic smokers. In view of the fact that structural cells, in particular fibroblasts release IL-6 and TGF-β1 in response to exposure to CSE ex vivo, and that both of these factors are known to promote Th17 T cell differentiation (Ivanov et al., 2006), we postulate that the main source of IL-17A is Th17 cells. IL-17A in turn stimulates structural cells of the airways (namely epithelial cells, endothelial cells, fibroblasts and airway smooth muscle cells) to release IL-8 a neutrophil chemoattactant (Laan et al., 1999, Molet et al., 2001, Vanaudenaerde et al., 2003). Furthermore we have provided evidence that CSE interacts with IL-17A and environmental stimuli such as allergens further to enhance expression of IL-6 and/or IL-8, so further supporting the development of IL-17A mediated neutrophilic inflammation (Figure 52)

In view of the fact that Esnault et al. have shown that treatment with mepolizumab (anti-IL5 antibody) results in a reduction in airway eosinophilia and IL-17A expression following segmental bronchial allergen challenge (Esnault et al., 2012), there is tenuous evidence to suggest that eosinophilic inflammation could induce IL-17A expression. Their data suggest that eosinophils induce IL-17A expression in CD4+ T cells through an IL-1β dependent mechanism. Further circumstantial evidence to support a role for eosinophils in supporting IL-17A production is our finding of a correlation between the degree of eosinophilia in the bronchial mucosa of asthmatics with the expression of IL-8, IL-17A and the degree of neutrophilia. In view of this we propose the tentative hypothesis that IL-17A mediated inflammation is at least partly propagated by eosinophils, while the action of cigarette smoke on the structural cells of the airways supports the perpetuation and further development of Th17/IL-17A mediated inflammation (Figure 52).

Our ex vivo findings suggest that CSE has the potential to support angiogenesis in the airways of smoking asthmatics by stimulating the release of VEGF by both fibroblasts and epithelial cells. We did not however uncover any evidence of increased angiogenesis in the bronchial mucosa of asthmatic smokers compared to non-smokers

in vivo in bronchial biopsies. It is our view that we were not able to detect an increase in angiogenesis as our study lacked the power in these very mild asthmatics with limited smoking history to detect small changes in airways remodelling (as discussed below in section 6.1.3) (Figure 53).

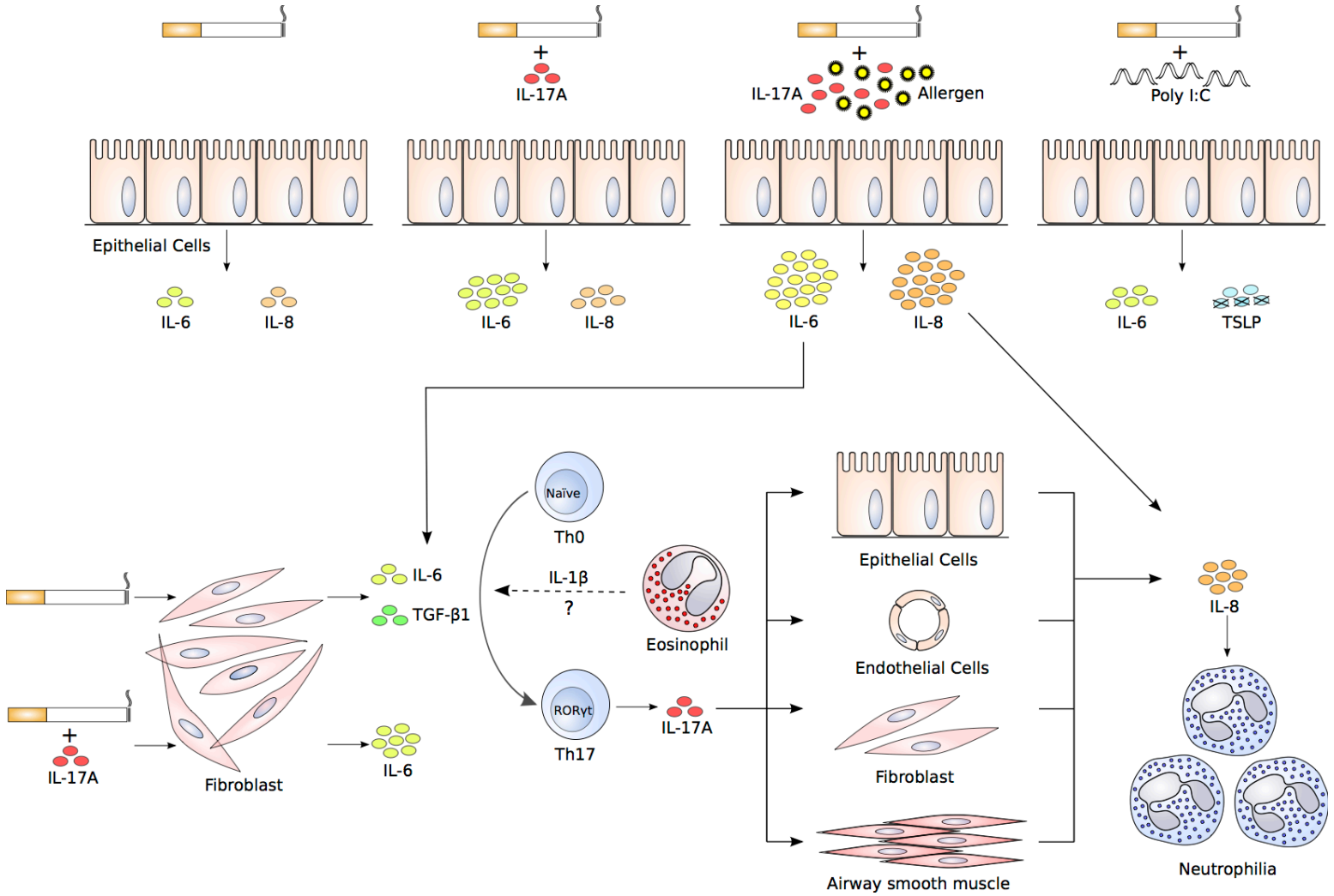

Figure 52: Th17/IL-17A mediated neutrophilic inflammation in asthmatic smokers

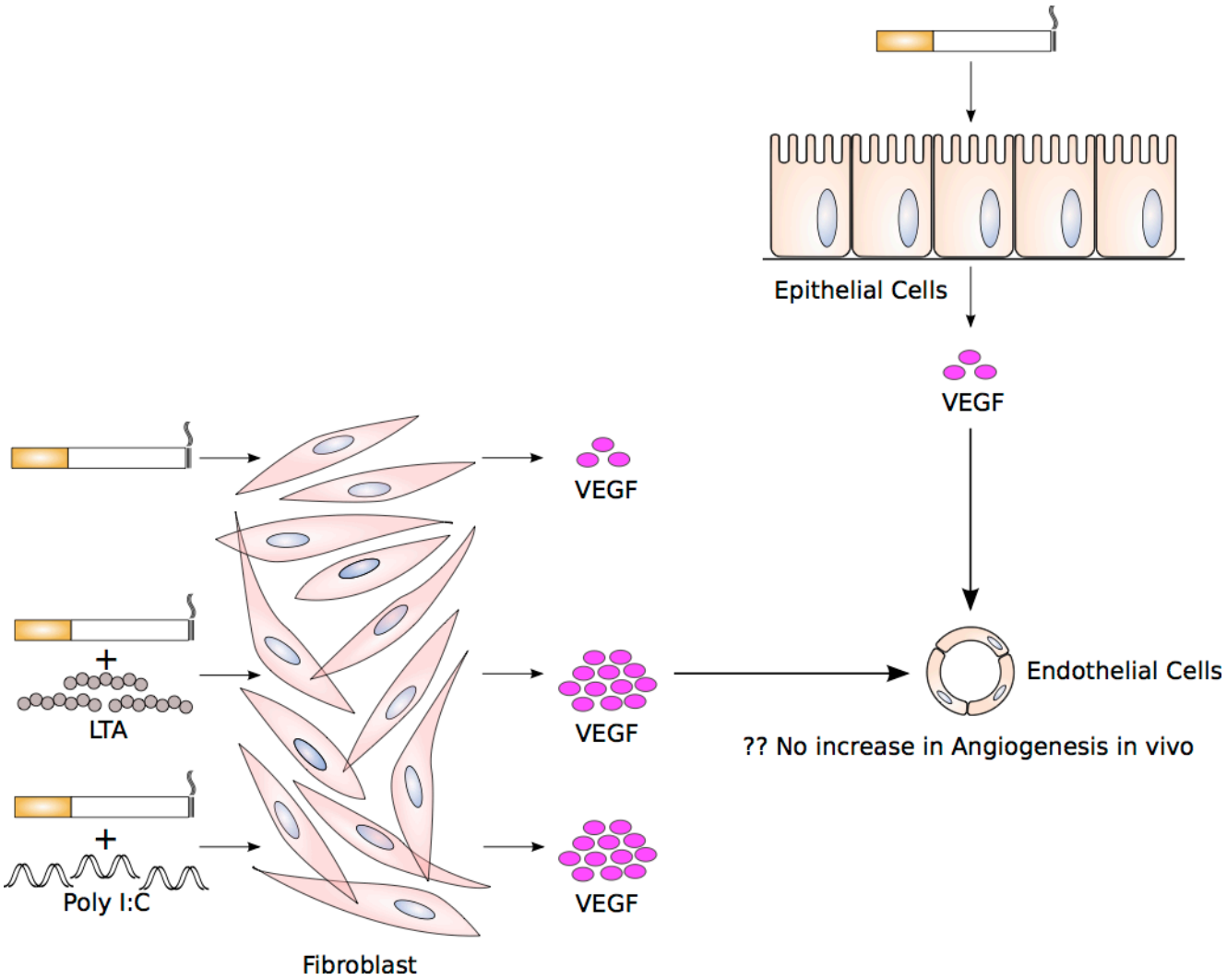

Figure 53: Cigarette smoking and vascular endothelial growth factor expression in asthmatic smokers

6.1.3 Limitations

This study investigating the inflammatory profile and vascular remodelling in smoking asthmatics was powered as a pilot study, since when it was commenced there were no suitable publications available which would provide data with which to power it. During the study period there were difficulties with recruiting steroid naïve smoking and non-smoking asthmatics, hence the relatively small numbers recruited, which has limited the power of the study to detect small changes in vascular remodelling which could be present in asthmatic smokers compared to non-smokers. Performing a sample size calculation with our data, using a power of 80% and alpha of 0.05, we would require a sample size in each asthmatic group of 18 and 46 to detect a significant difference in VEGF and vessel numbers respectively. As an entry criterion into the study, asthmatics were required to be steroid naïve to control for potential confounding effects of inhaled corticosteroids. It was important for us to control for this confounding factor in view of the prevailing evidence which suggests that inhaled corticosteroids are effective in reducing vascular remodelling in asthma (Hoshino et al., 2001b, Feltis et al., 2007). This necessitated recruiting a population of asthmatic smokers and non-smokers that were mild in severity with, putatively, a low degree of vascular remodelling. Alternatively one could have recruited more severe patients taking inhaled corticosteroids, introducing a further potential confounding factor. This is an age-old dilemma which is difficult to solve. In further studies one would wish to match the subjects for treatment and duration of disease, and stratify for severity. Longitudinal studies comparing smoking and non-smoking asthmatics would be useful. In view of the fact that we are comparing steroid naïve mild asthmatics who are smokers and non-smokers, our study is also likely to be underpowered to detect any differences in quality of life and symptoms.

With regard to the *in vitro* experiments, we used changes in the degree of confluence and trypan blue staining to assess the viability of the cultured adherent cells. This method is a relatively crude surrogate marker of viability and possibly does not reflect the true effect of CSE on cellular viability. Nevertheless our experiments were not designed to investigate the effects of CSE on cellular viability per se, and we performed experiments with concentrations of CSE that did not obviously affect the viability of cells. In view of this we felt that our assessment of cellular viability,

although relatively crude was sufficient for our experiments. We tried to assess viability with annexin-V/PI but encountered technical issues with cellular clumping that would have rendered the data uninterpretable. We did not use the gold standard MTT assay as this would have limited us to culturing the cells in 96 well plates. Despite all these reservations, our data on viability however showed a similar profile to those in published literature on the effects of CSE on cellular viability that was assessed by MTT assay (Yang et al., 2013, Baglole et al., 2006, Park et al., 2010).

Another limitation of these studies is the difficulty in equating exposure of airways structural cells such as epithelial cells and fibroblasts from "real life" smoking to cigarette smoke products with exposure to CSE *in vitro*. Exposure of cells from 24 hours up to 72 hours to CSE is obviously not a true reflection of what happens *in vivo* as even chain smokers do not smoke for 24 hours and up to 72 hours continuously. In view of the limitations of our *in vitro* experiments we are duly cautious in directly applying these *in vitro* results to represent reality *in vivo*. However the *in vitro* results allows us to better understand our *in vivo* findings in asthmatic smokers.

6.2 Clinical Implications

6.2.1 Endotyping in asthma

In 2008, Anderson published a paper on the classification of asthma based on endotypes, which are subsets of disease defined functionally and pathologically by a molecular mechanism or by treatment response (Anderson, 2008). Since then Lötvall et al. (Lotvall et al., 2011) and Wenzel (Wenzel, 2012) have proposed several endotypes which could be used to characterise asthma. The endotypes proposed by Lötvall et al. were aspirin sensitive asthma, allergic bronchopulmonary mycosis (ABPM), allergic asthma (adults), asthma-predictive indices positive preschool wheezer, severe late-onset hypereosinophilic, and asthma in cross country skiers (Lotvall et al., 2011). Those proposed by Wenzel were early onset allergic, persistent eosinophilia, ABPM, obese female and neutrophilic (Wenzel, 2012). Our data, along with published literature, indicate that asthmatic smokers do not fall within the neutrophilic endotype suggested by Wenzel and suggest that asthmatic smokers could be defined as a separate endotype with the following features:

- Clinical characteristics – poorly controlled asthma symptoms, frequent exacerbations and increased deterioration in lung function.
- Genetics – Th2 pathway with possible involvement of Th17 pathway
- Biomarkers – Unknown
- Epidemiology – tobacco or cigarette smoker, 25% of the asthma population
- Histopathology – Eosinophilic with neutrophilic inflammation in the bronchial mucosa with minimal difference in airways remodelling compared to asthma non-smokers.
- Response to treatment – Poor response to inhaled glucocorticoid treatment
- Proposed mechanism – Th17/IL-17A mediated neutrophilic inflammation overlying Th2 mediated eosinophilic inflammation

6.2.2 Steroid resistance in asthma smokers

Inhaled corticosteroids are an effective treatment for mild to moderate asthma, improving lung function and asthma control as well as reducing asthma exacerbations (1975, Smith and Hodson, 1983, Djukanovic et al., 1992, Fabbri et al., 1993). They have been shown to reduce airways inflammation (Djukanovic et al., 1992, Laitinen et al., 1992) and the changes associated with airways remodelling in patients with asthma (Laitinen et al., 1997, Hoshino et al., 1998b, Hoshino et al., 2001b, Ward et al., 2002, Feltis et al., 2007, James et al., 2012). In severe asthma treatment with inhaled corticosteroids alone is insufficient to control symptoms. A stepwise approach is used to increase treatment in order to achieve asthma control, although some patients remain symptomatic despite taking oral corticosteroids (BTS and SIGN, 2012, GINA, 2012). Patients with severe asthma have been suggested to be relatively insensitive to corticosteroid therapy (Carmichael et al., 1981, Hew et al., 2006, Bhavsar et al., 2008). Furthermore unlike mild to moderate asthmatics they also demonstrate a pattern of neutrophilic inflammation in their airways, although the underlying mechanism is poorly understood (is the neutrophilia a result of uncontrolled eosinophilic inflammation or is it partly a consequence of chronic exposure to corticosteroids?) (Wenzel et al., 1997). A similar pattern of neutrophilic inflammation is also seen in asthmatic smokers. However unlike severe asthma, asthmatic smokers do not show evidence of extensive airways "remodelling" despite also demonstrating a similar resistance to corticosteroid therapy and airway neutrophilia (Chalmers et al., 2001, Chalmers et al., 2002, Chaudhuri et al., 2003, Boulet et al., 2006, St-Laurent et al., 2008, Broekema et al., 2009). One potential reason for the corticosteroid insensitivity observed in severe asthma and smoking asthmatics could be the presence of neutrophilic inflammation in the airways. There is growing evidence that Th17 cells may orchestrate the neutrophilic inflammation seen in asthma, and our data reinforce this possibility.

In an ovalbumin (OVA) sensitized mouse model of asthma, neutrophilic infiltration of the airways following an acute allergen challenge was found to be dependent on IL-17A expression (Hellings et al., 2003). Furthermore mice that were sensitised and had prolonged exposure to OVA were shown to have an increased number of Th17 cells infiltrating into the airways and this was positively correlated with the extent of

airways remodelling. Adoptive transfer of sensitised Th17 cells resulted in an acceleration in the development of airways remodelling, while neutralisation of IL-17A (using a monoclonal anti-IL-17A antibody) resulted in attenuation of this remodelling (Wang et al., 2010). Surprisingly anti-IL-17A monoclonal antibody enhanced OVA induced eosinophilic infiltration of the airways despite inhibiting neutrophilic infiltration (Hellings et al., 2003). Transgenic mice overexpressing retinoic acid receptor-related orphan receptor gamma t (RORγt, a key transcription factor for Th17 differentiation) developed an OVA-induced neutrophilic infiltration of the airways which was insensitive to corticosteroid reversal (Ano et al., 2013).

In patients with severe asthma there is increased expression of IL-17A in induced sputum, which correlates with the expression of IL-8 and the percentages of neutrophils (Sun et al., 2005). Furthermore steroid resistant moderate-severe asthmatics have been found to have elevated percentages of IL-17A and IL-22 expressing cells in peripheral blood mononuclear cells (PBMC) depleted of $CD8^+$ T cells and expanded *ex vivo* with anti-CD3 and IL-2. Co-culture of these cells with dexamethasone resulted in a tendency for increased secretion of IL-17A (Nanzer et al., 2013). A similar increase in IL-17A expression has been shown in smoking asthmatics treated with oral dexamethasone, where corticosteroid treatment resulted in elevated expression of IL-17A in induced sputum (Spears et al., 2013). In blood mononuclear cells, the combination of IL-17A and IL-17F co-stimulation increases the expression of glucocorticoid receptor(GR)-beta, while the combination of IL-2 and IL-4 co-stimulation reduces the expression of GR-alpha. These two combinations of stimuli increased the GR-beta/GR-alpha ratio and resulted in an insensitivity to dexamethasone induced early apoptosis and inhibition of proliferation (Vazquez-Tello et al., 2013).

Our findings of increased expression of IL-17A in the bronchial mucosa of smoking asthmatics and that CSE synergistically interacts with IL-17A provide new and supportive evidence concerning the growing body of literature implicating neutrophilic inflammation in asthma and corticosteroid resistance, particularly in the context of asthmatic smokers.

6.2.3 Implications for future therapies

Despite the efforts of governments to encourage smoking cessation, approximately a quarter of all asthmatics globally are current smokers, and indeed the overall prevalence of smoking in asthmatics is no different to that of the general population (To et al., 2012b). Furthermore asthmatic smokers have poor symptom control, increased exacerbations and an accelerated decline in lung function (Althuis et al., 1999, James et al., 2005, Boulet et al., 2006, Eisner and Iribarren, 2007, Chaudhuri et al., 2008). Since they demonstrate insensitivity to both inhaled and oral corticosteroid therapy (Chalmers et al., 2002, Chaudhuri et al., 2003) there is an unmet need for the development of new modalities of treatment.

We have demonstrated that there is increased expression of IL-17A in the bronchial mucosa of asthmatic smokers and that IL-17A and cigarette smoke can interact in a synergistic manner with structural cells, causing the release of pro-inflammatory cytokines. Furthermore Spears at al. (Spears et al., 2013) have shown that treatment with oral dexamethasone causes an increase expression of IL-17A in smoking asthmatics. We therefore postulate that a possible target to attenuate insensitivity to corticosteroids seen in smoking asthmatics is Th17 cells and the pro-inflammatory mediators they release.

RORγt is they key transcription factor for Th17 differentiation and its expression is up regulated in the presence of both IL-6 and TGF-β1 (Ivanov et al., 2006). Up regulation of RORγt by IL-6 and TGF-β1 alone is insufficient for Th17 differentiation in humans (Chen et al., 2007). In naïve $CD4^+$ T cells, IL-6, TGF-β1 and IL-23 are required for Th17 differentiation and IL-17A expression (Ganjalikhani Hakemi et al., 2011). Memory $CD4^+$ T cells however only require the presence of IL-23 in order to differentiate into Th17 cells, following activation with CD3/CD28 stimulation (Chen et al., 2007). Of note IL-23R has been shown to be absent on resting naïve $CD4^+$ T cells and T cell activation resulted in increased expression of IL-23R (Vanden Eijnden et al., 2005).

In view of this a monoclonal anti-IL-23 antibody might be useful in attenuating T cell polarisation towards a Th17 phenotype and therefore reduce the insensitivity to corticosteroids seen in asthmatic smokers. In mouse models of autoimmune diseases monoclonal anti-IL-23 (anti-IL23p19) antibody has been shown to reduce IL-17A expression, inhibiting the development of experimental autoimmune encephalomyelitis (Chen et al., 2006) and reducing the disease score in collagen-induced arthritis (Cornelissen et al., 2013). IL-6 blockade (using either a monoclonal antibody to IL-6 or IL-6R) has been shown to be effective in ameliorating autoimmune diseases in a variety of murine models by inhibiting the development of Th17 cells (Serada et al., 2008, Fujimoto et al., 2008, Aricha et al., 2011). Despite the differing importance of IL-23 in the development of Th17 cells in mice and humans, IL-6 blockade using a monoclonal antibody to IL-6 receptor (tocilizumab) has been shown to be effective in reducing the percentages of Th17 cells while also increasing the percentage of Treg cells in the peripheral blood of patients with rheumatoid arthritis. These changes were associated with an improvement in disease activity score (Samson et al., 2012). There is therefore a potential for a role for monoclonal antibodies that block either IL-23 or IL-6 to be able to reduce the IL-17A mediated inflammatory response in asthmatic smokers and non-smoking severe asthmatics. This may in turn reduce airway neutrophilia and possibly insensitivity to corticosteroids.

A randomized, placebo controlled trial investigating brodalumab, an anti-IL-17RA monoclonal antibody, in inadequately controlled moderate-severe asthma patients suggested that blockade of IL-17RA did not significantly improve asthma control and lung function (Busse et al., 2013). A similar situation pertained in patients with methotrexate-resistant rheumatoid arthritis, where brodalumab was found to be ineffective at improving disease control (Martin et al., 2013). Nevertheless, neutralisation of IL-17A has been shown to be effective in improving disease control and activity in patients with rheumatoid arthritis and psoriasis, with a reduction in the number of $IL\text{-}17A^{+}CD3^{+}$ T cells in psoriasis plaques (Genovese et al., 2010, Hueber et al., 2010). A monoclonal anti-IL-17A antibody may therefore be potentially effective in attenuating the IL-17A mediated neutrophilic inflammation seen in smoking asthmatics.

6.3 Future directions

We have shown elevated numbers of IL-17A positive cells in the bronchial mucosa of smoking asthmatic patients. Even though Th17 T cells coordinate inflammatory responses by the release of IL-17A (as well as IL-17F and IL-22), several other inflammatory cells (γδT cells, invariant NKT cells, innate lymphoid cells, mast cells and neutrophils) have also been shown to express IL-17A in response to a variety of stimuli (Lockhart et al., 2006, Michel et al., 2007, Buonocore et al., 2010, Lin et al., 2011). In view of this the next step forward would be to investigate the precise cellular source of IL-17A in the bronchial mucosa of asthmatic smokers. This could be approached either by double staining immunohistochemistry of endobronchial biopsy sections or by flow cytometric analysis of the cells in bronchoalveolar lavage fluid from asthmatic smokers.

IL-23 is an important cytokine required for the differentiation of naïve T cells in to Th17 cells (Chen et al., 2007, Ganjalikhani Hakemi et al., 2011). We did not investigate whether there was increased expression of IL-23 in the bronchial mucosa of asthmatic smokers in this project primarily because we were investigating the interplay between structural cells, cigarette smoke and the Th17/IL-17A inflammatory axis in asthmatic smokers. It has yet to be determined whether there is increased expression of IL-23 in the airways of asthmatic smokers and whether structural cells are capable of expressing IL-23; the main sources of IL-23 currently identified are antigen-presenting cells (Pirhonen et al., 2002, Sheibanie et al., 2004). This could be achieved by immunohistochemical staining of endobronchial biopsy sections and/or measurement of the cytokine concentrations in induced sputum from asthmatic smokers.

The mechanism by which cigarette smoking increases the expression of IL-17A in the bronchial mucosa could also be studied using animal models of asthma. Transgenic C57BL/6 RORγ knockout mice sensitized to OVA and exposed to OVA and cigarette smoke could be used to investigate the importance of Th17 cells in mediating the neutrophilic inflammation seen in asthmatic smokers (Morokata et al., 1999) (Ivanov et al., 2006). The importance of IL-17A in mediating the neutrophilic inflammation in

smoking asthmatics might be elucidated using transgenic C57BL/6 IL-17A knockout mice sensitized to OVA and exposed to OVA and cigarette smoke (Morokata et al., 1999, Schulz et al., 2008). Analysis of bronchoalveolar lavage and lung tissue sections from these murine models might further clarify the importance of Th17 cells and IL-17A in the neutrophilic inflammation seen in asthmatic smokers.

Loubaki et al. (Loubaki et al., 2013) showed that co-culture of human bronchial fibroblasts with $CD4^+$ T cells augmented T cell differentiation to Th17 cells, particularly in the presence of fibroblasts with the asthma phenotype. In view of the findings of Loubaki et al. and our findings that CSE is able to induce the release of IL-6 and TGFβ-1 in primary bronchial fibroblasts, it would be interesting to extend both our findings and those of Loubaki et al. by investigating whether CSE is able synergistically or additively to influence the development of $CD4^+$ T cells into Th17 cells by co-culture with primary bronchial fibroblasts, which might further highlight the importance of bronchial fibroblasts in the development of IL-17A mediated inflammation in asthmatic smokers.

Another investigative route would be to stimulate either PBMC or purified $CD4^+$ T cells with anti-CD3, anti-CD28 and IL-2 in the presence and absence of CSE. The polyclonally stimulated and expanded cells could be harvested at 24 hours and at 7 days following stimulation. Flow cytometric analysis could be used to determine the proportion of Th17 cells before and after stimulation to determine if CSE is able to promote T cell differentiation into Th17 cells following polyclonal stimulation. The advantage of using PBMCs rather than purified $CD4^+$ T cells is that the supernatants in a PBMC culture model could be harvested at the same time points to measure the release of IL-6, TGFβ-1 and IL-23, and the effect of CSE on the release of these mediators by antigen presenting cells and myeloid cells could be investigated and provide supporting mechanistic data should it be the case that CSE does indeed induce T cells to differentiate into Th17 cells.

Our findings that CSE reduces Poly I:C mediated release of TSLP by HTEpC warrants further investigation as this result implies that CSE could preferentially inhibit allergic inflammation mediated via TSLP expression. Endobronchial biopsy

sections from asthmatic smokers and non-smokers could be stained for TSLP expression. This method however has limitations as TSLP is expressed primarily by bronchial epithelial cells (Ying et al., 2005), and epithelial desquamation in the airways of asthmatic smokers and non-smokers is seen in endobronchial biopsies (St-Laurent et al., 2008). In view of this the results from immunohistochemical staining could be difficult to interpret. There is increasing evidence to suggest that periostin may be used as a biomarker for allergic inflammation in asthma (Sidhu et al., 2010, Jia et al., 2012, Masuoka et al., 2012, Kanemitsu et al., 2013). One could therefore use periostin as a surrogate marker of Th2 inflammation and measure periostin expression in the peripheral blood or endobronchial biopsy sections from asthmatic smokers and non-smokers. In view of the fact that periostin is yet to be fully validated as a biomarker any results from these experiments should be considered exploratory in nature.

Our data imply that the synergistic and additive interaction between IL-17A and CSE to stimulate epithelial cells to release the pro-inflammatory mediator IL-6 is mediated by the generation of reactive oxygen species. The mechanism for this interaction warrants further investigation with the use of intracellular inhibitors, small interfering RNA (siRNA) and antioxidants. The kinetics of the interaction between IL-17A and CSE to generate reactive oxygen species could be investigated using fluorescent/luminescent probes (Chen et al., 2011). This investigation along with the measurement of phosphorylation of various key intracellular signalling mediators (identified using intracellular inhibitors or siRNA) would allow us better to understand the mechanism behind the synergistic/additive interaction between IL-17A and CSE. The mechanism behind the synergistic interaction between IL-17A, CSE and allergen could be investigated in a similar manner.

6.4 Conclusion

In conclusion, the data in this thesis demonstrate that in asthmatic smokers there is IL-17A mediated inflammation, and suggest that IL-17A is at least one key mediator driving the neutrophilic inflammation seen in asthmatic smokers. The data also clearly show that structural cells potentially play an important role in the development and maintenance of cigarette smoke induced IL-17A mediated inflammation in asthmatic smokers. These findings suggest that asthmatic smokers are a separate endotype of asthma and more research is necessary to ensure that there are more effective therapies available for asthmatics who cannot stop smoking.

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